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DESCRIPITION

HISTONE DEACETYLASE INHIBITORS AND METHODS FOR PRODUCING THE SAME

Technical Field

The present invention relates to histone deacetylase (HDAC) inhibitors and methods for producing the same.

Background Art

Eukaryotic chromatin structure and gene expression are regulated by histone acetylation by histone acetyltransferase (HAT), and deacetylation by histone deacetylase (HDAC). HDAC inhibitors are already known to induce cancer cell differentiation and apoptosis, and are expected to be useful as antitumor agents (Marks, P. A., Richon, V. M., and Rifkind, R. A. (2000). Histone deacetylase inhibitors: Inducers of differentiation or apoptosis of transformed cells. J. Natl. Cancer Inst. 92, 1210-1216; Yoshida, M., Horinouchi, S., and Beppu, T. (1995). Trichostatin A and trapoxin: novel chemical probes for the role of histone acetylation in chromatin structure and function. Bioessays 17, 423-430; Bernhard, D., Löffler, M., Hartmann, B. L., Yoshida, M., Kofler, R., and Csordas, A. (1999). Interaction between dexamethasone and butyrate in apoptosis induction: non-additive in thymocytes and synergistic in a T cell-derived leukemia cell line. Cell Death Diff. 6, 609-607).

In fact, clinical studies have begun in the United States for some HDAC inhibitors (Nakajima, H., Kim, Y. B., Terano, H., Yoshida, M., and Horinouchi, S. (1998). FR901228, a potent antitumor antibiotic, is a novel histone deacetylase inhibitor. Exp. Cell Res. 241, 126-133; Saito, A., Yamashita, T., Mariko, Y., Nosaka, Y., Tsuchiya, K., Ando, T., Suzuki, T., Tsuruo, T., and Nakanishi, O. (1999). A synthetic inhibitor of histone deacetylase, MS-27-275, with marked *in vivo* antitumor activity against human tumors. Proc. Natl. Acad. Sci. USA 96, 4592-4597) that are effective as antitumor agents in animal experiments.

Tricostatin A (TSA) is well known as a specific HDAC inhibitor (Yoshida, M., Kijima, M., Akita, M., and Beppu, T. (1990). Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by trichostatin A. J. Biol. Chem. 265, 17174-17179). Actually, TSA has been known to induce differentiation to leukemia cells, neuronal cells, breast cancer cells, and the like (Yoshida, M., Nomura, S., and Beppu, T. Effects of trichostatins on differentiation of murine erythroleukemia cells. Cancer Res. 47: 3688-3691, 1987; Hoshikawa, Y., Kijima, M., Yoshida, M., and Beppu, T. Expression of differentiation-related markers in teratocarcinoma cells via histone hyperacetylation by trichostatin A. Agric. Biol. Chem. 55: 1491-1495, 1991; Minucci, S., Horn, V., Bhattacharyya, N., Russanova, V., Ogryzko, V. V., Gabriele, L., Howard, B. H., and Ozato, K. A histone deacetylase inhibitor potentiates retinoid receptor action in embryonal carcinoma cells. Proc. Natl. Acad. Sci. USA 94: 11295-11300, 1997; Inokoshi, J., Katagiri, M., Arima, S., Tanaka, H., Hayashi, M., Kim, Y. B., Furumai, R., Yoshida, M., Horinouchi, S., and Omura, S. (1999). Neuronal differentiation of Neuro 2a cells by inhibitors of cell progression, trichostatin A and butyrolactone I. Biochem. Biophys. Res. Commun. 256, 372-376; Wang, J., Saunthararajah, Y., Redner, R. L., and Liu, J. M. Inhibitors of histone deacetylase relieve ETO-mediated repression and induce differentiation of AML1-ETO leukemia cells. Cancer Res. 59: 2766-2769, 1999; Munster, P. N., Troso-Sandoval, T., Rosen, N., Rifkind, R., Marks, P. A., and Richon, V. M. The histone deacetylase inhibitor suberoylanilide hydroxamic acid induces differentiation of human breast cancer cells. Cancer Res. 61: 8492-8497, 2001; Ferrara, F. F., Fazi, F., Bianchini, A., Padula, F., Gelmetti, V., Minucci, S., Mancini, M., Pelicci, P. G., Lo Coco, F., and Nervi, C. Histone deacetylase-targeted treatment restores retinoic acid signaling and differentiation in acute myeloid leukemia. Cancer Res. 61: 2-7, 2001; Gottlicher, M., Minucci, S., Zhu, P., Kramer, O. H., Schimpf, A., Giavara, S., Sleeman, J. P., Lo Coco, F., Nervi, C., Pelicci, P. G., and

Heinzel, T. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. EMBO J. 20: 6969-6978, 2001). Furthermore, the TSA activities of differentiation induction and apoptosis induction are known to synergistically increase when used in combination with drugs which activate gene expression by mechanisms different to HDAC inhibitors. For example, cancer cell differentiation is promoted by using HDAC inhibitors in combination with retinoic acids, which activate retinoic acid receptors that serve as nuclear receptors, inducing gene expression relevant to differentiation (Minucci, S., Horn, V., Bhattacharyya, N., Russanova, V., Ogryzko, V. V., Gabriele, L., Howard, B. H., and Ozato, K. A histone deacetylase inhibitor potentiates retinoid receptor action in embryonal carcinoma cells. Proc. Natl. Acad. Sci. USA 94: 11295-11300, 1997; Ferrara, F. F., Fazi, F., Bianchini, A., Padula, F., Gelmetti, V., Minucci, S., Mancini, M., Pelicci, P. G., Lo Coco, F., and Nervi, C. Histone deacetylase-targeted treatment restores retinoic acid signaling and differentiation in acute myeloid leukemia. Cancer Res. 61: 2-7, 2001; Coffey, D. C., Kutko, M. C., Glick, R. D., Butler, L. M., Heller, G., Rifkind, R. A., Marks, P. A., Richon, V. M., and La Quaglia, M. P. The histone deacetylase inhibitor, CBHA, inhibits growth of human neuroblastoma xenografts in vivo, alone and synergistically with all-trans retinoic acid. Cancer Res. 61: 3591-3594, 2001; Petti, M. C., Fazi, F., Gentile, M., Diverio, D., De Fabritiis, P., De Propris, M. S., Fiorini, R., Spiriti, M. A., Padula, F., Pelicci, P. G., Nervi, C., and Lo Coco, F. Complete remission through blast cell differentiation in PLZF/RARalpha-positive acute promyelocytic leukemia: in vitro and in vivo studies. Blood 100: 1065-1067, 2002). 5-azadeoxycytidine inhibits DNA methylation to reduce expression of tumor suppressor genes in many cancer cells. TSA used in combination with 5-azadeoxycytidine promotes cancer cell apoptosis and restoration of tumor-suppressing gene expression (Nan, X., Ng, H. H., Johnson, C. A., Laherty, C. D., Turner, B. M., Eisenman, R. N., and Bird, A. Transcriptional repression by

deacetylase complex. Nature 393: 386-389, 1998; Cameron, E. E., Bachman, K. E., Myohanen, S., Herman, J. G., and Baylin, S. B. Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nature Genet. 21: 103-107, 1999; Li, Q. L., Ito, K., Sakakura, C., Fukamachi, H., Inoue, K., Chi, X. Z., Lee, K. Y., Nomura, S., Lee, C. W., Han, S. B., Kim, H. M., Kim, W. J., Yamamoto, H., Yamashita, N., Yano, T., Ikeda, T., Itohara, S., Inazawa, J., Abe, T., Hagiwara, A., Yamagishi, H., Ooe, A., Kaneda, A., Sugimura, T., Ushijima, T., Bae, S. C., and Ito, Y. Causal relationship between the loss of RUNX3 expression and gastric cancer. Cell 109: 113-124, 2002; Boivin, A. J., Momparler, L. F., Hurtubise, A., and Momparler, R. L. Antineoplastic action of 5-aza-2'-deoxycytidine and phenylbutyrate on human lung carcinoma cells. Anticancer Drugs 13: 869-874, 2002; Primeau, M., Gagnon, J., and Momparler, R. L. Synergistic antineoplastic action of DNA methylation inhibitor 5-AZA-2'-deoxycytidine and histone deacetylase inhibitor depsipeptide on human breast carcinoma cells. Int J Cancer 103: 177-184, 2003).

HDAC inhibitors are expected to be not only antitumor agents but also cancer preventives. TSA, SAHA, and the like significantly suppressed the occurrence of breast cancer induced in animal models. Also, investigations carried out using valproic acids indicated that HDAC inhibitors suppress metastasis (Gottlicher, M., Minucci, S., Zhu, P., Kramer, O. H., Schimpf, A., Giavara, S., Sleeman, J. P., Lo Coco, F., Nervi, C., Pelicci, P. G., and Heinzel, T. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. EMBO J. 20: 6969-6978, 2001).

HDAC inhibitors are used not only as tumor suppressive agents, but also, for example, as agents for treating and improving autoimmune diseases, skin diseases, infectious diseases, and such (Darkin-Rattray et al. Proc. Natl. Acad. Sci. USA 93, 13143-13147, 1996), as well as in improving the efficiency of vector introduction in gene therapy (Dion et al., Virology 231, 201-209, 1997), promoting the expression of

introduced genes (Chen et al., Proc. Natl. Acad. Sci. USA 94, 5798-5803, 1997), and the like. Furthermore, HDAC inhibitors are presumed to have angiogenesis-inhibiting functions (Kim, M. S., Kwon, H. J., Lee, Y. M., Baek, J. H., Jang, J. E., Lee, S. W., Moon, E. J., Kim, H. S., Lee, S. K., Chung, H. Y., Kim, C. W., and Kim, K. W. (2001). Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes. Nature Med. 7, 437-443; Kwon, H. J., Kim, M. S., Kim, M. J., Nakajima, H., and Kim, K. W. (2002). Histone deacetylase inhibitor FK228 inhibits tumor angiogenesis. Int. J. Cancer 97, 290-296).

Ten or more HDAC subtypes exist, and recently, specific HDAC subtypes have been identified as being closely related to cancers. For example, it has been discovered that acetylation of the tumor suppressor gene p53, which plays an extremely important role in suppressing carcinogenesis, is very important in the functional expression of p53 itself (Ito, A., Lai, C. H., Zhao, X., Saito, S., Hamilton, M. H., Appella, E., and Yao, T. P. (2001). p300/CBP-mediated p53 acetylation is commonly induced by p53-activating agents and inhibited by MDM2. EMBO J. 20, 1331-1340), and HDAC1 and HDAC2 are involved in the inhibition of p53 function (Juan, L. J., Shia, W. J., Chen, M. H., Yang, W. M., Seto, E., Lin, Y. S., and Wu, C. W. (2000). Histone Deacetylases Specifically Down-regulate p53-dependent Gene Activation. J. Biol. Chem. 275, 20436-20443). It has also been discovered that proteins PML-RAR and PLZF-RAR, involved in the onset of promyelocytic leukemia (APL), and oncogenes such as Bcl-6, which is involved in the onset of lymphomas, recruit HDAC4 or such via nuclear co-repressors, and suppress expression of the gene group necessary for normal differentiation, causing carcinogenesis (Dhordain P., Albagli, O., Lin, R. J., Ansieau, S., Quief, S., Leutz, A., Kerckaert, J. P., Evans, R. M., and Leprince, D. (1997). Corepressor SMRT binds the BTB/POZ repressing domain of the LAZ3/BCL6 oncoprotein. Proc. Natl. Acad. Sci. USA 94, 10762-10767; Grignani, F., De, M. S., Nervi, C., Tomassoni, L., Gelmetti, V., Cioce, M., Fanelli, M., Ruthardt, M., Ferrara, F. F., Zamir, I., Seiser, C., Grignani, F., Lazar, M. A., Minucci,

S., and Pelicci, P. G. (1998). Fusion proteins of the retinoic acid receptor-alpha recruit histone deacetylase in promyelocytic leukaemia. Nature 391, 815-818; He, L. Z., Guidez, F., Tribioli, C., Peruzzi, D., Ruthardt, M., Zelent, A., and Pandolfi, P. P. (1998). Distinct interactions of PML-RARalpha and PLZF-RARalpha with co- repressors determine differential responses to RA in APL. Nature Genet. 18, 126-135; Lin, R. J., Nagy, L., Inoue, S., Shao, W., Miller, W. J., and Evans, R. M. (1998). Role of the histone deacetylase complex in acute promyelocytic leukaemia. Nature 391, 811-814). On the other hand, HDAC subtypes which play a very important role in the development and differentiation of normal tissues are known to exist among those HDAC subtypes with tissue-specific expression (McKinsey, T. A., Zhang, C. L., Lu, J., and Olson, E. N. (2000). Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. Nature 408, 106-111; Verdel, A., and Khochbin, S. (1999). Identification of a new family of higher eukaryotic histone deacetylases. Coordinate expression of differentiation-dependent chromatin modifiers. J. Biol. Chem. 274, 2440-2445). In order to avoid inhibition of these HDACs, development of a subtype-specific inhibitor is thought to be necessary.

HDAC6 is an enzyme which is shuttled between the nucleus and the cytoplasm by nucleo-cytoplasmic transport, and which normally locates in the cytoplasm (Verdel, A., Curtet, S., Brocard, M.-P., Rousseaux, S., Lemercier, C., Yoshida, M., and Khochbin, S. (2000). Active maintenance of mHDA2/mHDAC6 histone-deacetylase in the cytoplasm. Curr. Biol. 10, 747-749). HDAC6 is highly expressed in the testes, and is presumed to relate to the differentiation of normal tissues. Furthermore, HDAC6 is known to be associated with microtubule deacetylation, and to control microtubule stability (Matsuyama, A., Shimazu, T., Sumida, Y., Saito, A., Yoshimatsu, Y., Seigneurin-Berny, D., Osada, H., Komatsu, Y., Nishino, N., Khochbin, S., Horinouchi, S., and Yoshida, M. (2002). In vivo destabilization of dynamic microtubules by HDAC6-mediated deacetylation. EMBO J. 21, 6820-6831). HDAC6 is also a deacetylation enzyme bonded to a

microtubule and affecting cell mobility (Hubbert, C., Guardiola, A., Shao, R., Kawaguchi, Y., Ito, A., Nixon, A., Yoshida, M., Wang, X.-F., and Yao, T.-P. (2002). HDAC6 is a microtubule-associated deacetylase. Nature 417, 455-458). Accordingly, HDAC6 inhibitors may be metastasis-suppressing agents. TSA inhibits each HDAC subtype to about the same degree. However, HDAC6 cannot be inhibited by trapoxins comprising cyclic tetrapeptide structure and epoxyketone as active groups (Furumai, R., Komatsu, Y., Nishino, N., Khochbin, S., Yoshida, M., and Horinouchi, S. Potent histone deacetylase inhibitors built from trichostatin A and cyclic tetrapeptide antibiotics including trapoxin. Proc. Natl. Acad. Sci. USA 98: 87-92, 2001). Based on the information on the three-dimensional structure of the enzyme, trapoxins are assumed to exert poor binding properties to HDAC6 due to the structure of its cyclic tetrapeptide moiety that interacts with the weakly conserved outward surface of the enzyme active center. This implies that altering the cyclic tetrapeptide portion may result in inhibitors that are selective for a variety of HDAC.

TSA shows inhibition activity due to the coordination of its hydroxamic acid group with zinc in the HDAC active pocket (Finnin, M. S., Donigian, J. R., Cohen, A., Richon, V. M., Rifkind, R. A., Marks, P. A., Breslow, R., and Pavletich, N. P. Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. Nature 401: 188-193, 1999). Examples of known HDAC inhibitors comprising hydroxamic acid are Oxamflatin (Kim, Y. B., Lee, K.-H., Sugita, K., Yoshida, M., and Horinouchi, S. Oxamflatin is a novel antitumor compound that inhibits mammalian histone deacetylase. Oncogene 18: 2461-2470, 1999) and CHAP (Furumai, R., Komatsu, Y., Nishino, N., Khochbin, S., Yoshida, M., and Horinouchi, S. Potent histone deacetylase inhibitors built from trichostatin A and cyclic tetrapeptide antibiotics including trapoxin. Proc. Natl. Acad. Sci. USA 98: 87-92, 2001., Komatsu, Y., Tomizaki, K.-y., Tsukamoto, M., Kato, T., Nishino, N., Sato, S., Yamori, T., Tsuruo, T., Furumai, R., Yoshida, M., Horinouchi, S., and Hayashi, H. Cyclic Hydroxamic-

acid-containing Peptide 31, a potent synthetic histone deacetylase inhibitor with antitumor activity. Cancer Res. 61: 4459-4466, 2001). However, since TSA is instable in blood and has a strong hydroxamic acid chelating function, it chelates with other essential metal ions, and therefore, HDAC inhibitors comprising hydroxamic acid have not actually been used as antitumor agents to date. Meanwhile, thiol groups produced by the reduction of FK228 disulfide bonds have recently been shown to serve as active groups to be coordinated with zinc in the HDAC active pocket, inhibiting HDAC. Thus, FK228 is a prodrug that is activated when reduced by cellular reducing activity (Furumai, R., Matsuyama, A., Kobashi, N., Lee, K.-H., Nishiyama, M., Nakajima, H., Tanaka, A., Komatsu, Y., Nishino, N., Yoshida, M., and Horinouchi, S. (2002). FK228 (depsipeptide) as a natural prodrug that inhibits class I histone deacetylases. Cancer Res. 62, 4916-4921).

Furthermore, a number of HDAC inhibitors comprising cyclic tetrapeptide structures and epoxyketones as active groups have been isolated from natural environments. On the basis of such findings, the cyclic tetrapeptide structure is suggested to be useful in enzyme identification (as described above, Yoshida, et al., 1995), however, from various viewpoints such as stability, existing inhibitors have not advanced to the level of being satisfactorily qualified as pharmaceutical products. Therefore, production of pharmaceutical agents in which these problematic points have been resolved is strongly anticipated.

Disclosure of the Invention

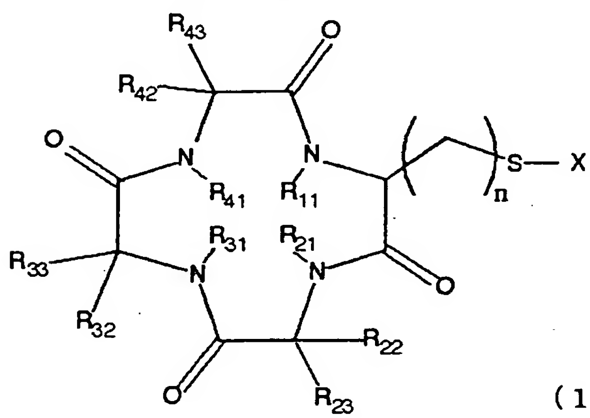
The present inventors aim to provide novel HDAC inhibitors comprising a cyclic tetrapeptide structure, and methods for producing the same.

In consideration of the above-mentioned objectives, the inventors of the present invention synthesized compounds comprising cyclic tetrapeptide structures that comprise thiol groups and their disulfide bonds, and then analyzed the HDAC inhibition activity of these compounds. As a result, it was

found that compounds comprising disulfide bonds did not exhibit very high HDAC inhibition activity against enzymes *in vitro*. However, when converted into thiols by coexisting with the reducing agent dithiothreitol, they showed strong HDAC inhibition activity. On the other hand, the intracellular level of disulfide activity was observed to be as high as that of TSA and thiols. Accordingly, disulfides were shown to be useful as prodrugs for HDAC inhibitors, in which the disulfide bonds are cleaved by intracellular reduction after being taken up into cells, inducing strong activity. Furthermore, the compounds were found to be more stabile in the serum when the thiol groups were protected in such a manner, and it was discovered that by binding the protection groups (-SX) with various functional compounds, the compounds could bind to compounds with desired activities, other than HDAC inhibitors.

The invention relates to HDAC inhibitors and methods for producing the same, and specifically provides the following [1] to [9]:

[1] A compound represented by the following formula (1):



(1)

[wherein, R_{11}, R_{21}, R_{31}, and R_{41} independently denote hydrogen or methyl; R_{22}, R_{23}, R_{32}, R_{33}, R_{42}, and R_{43} independently denote a hydrogen, a linear alkyl with one to six carbon atoms, a linear alkyl with one to six carbon atoms to which a non-aromatic cyclic alkyl group or substituted or unsubstituted aromatic ring, a non-aromatic cyclic alkyl, or a non-aromatic cyclic alkyl group to which a non-aromatic cyclic alkyl group or a substituted or unsubstituted aromatic ring is bound; the pairs

of R_{21} and R_{22}, R_{22} and R_{23}, R_{31} and R_{32}, R_{32} and R_{33}, R_{41} and R_{42}, and R_{42} and R_{43} independently denote acyclic structures without binding or cyclic structures by binding through a linear alkylene group with a one- to five-carbon main chain, a linear alkylene group with a one- to five-carbon main chain comprising a branched chain with a one to six carbons, or a linear alkylene group with a one- to five-carbon main chain comprising a ring structure of one to six carbons; X denotes hydrogen, a structure identical to that shown to the left of X, a substituted or unsubstituted alkyl or aryl group in any structure comprising a sulfur atom capable of binding with the sulfur atom in formula (1) through a disulfide bond, or a sulfur atom binding with the sulfur atom bonded to the terminus of R_{22}, R_{23}, R_{32}, R_{33}, R_{42}, or R_{43}, and located to the left of X, via an intramolecular disulfide bond].

[2] A histone deacetylase inhibitor that comprises the compound of [1] as an active ingredient.

[3] An apoptosis inducing agent that comprises the compound of [1] as an active ingredient.

[4] A differentiation-inducing agent that comprises the compound of [1] as an active ingredient.

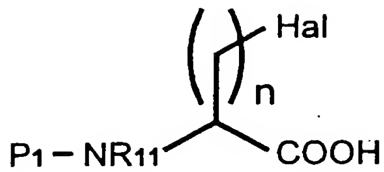
[5] An angiogenesis inhibitor that comprises the compound of [1] as an active ingredient.

[6] An anti-metastatic agent comprising the compound of [1] as an active ingredient.

[7] A pharmaceutical agent for treating or preventing a disease caused by histone deacetylase 1 or 4, comprising the compound of [1] as an active ingredient.

[8] The pharmaceutical agent of [7], wherein the disease caused by histone deacetylase 1 or 4 is cancer, autoimmune disease, skin disease, or infectious disease.

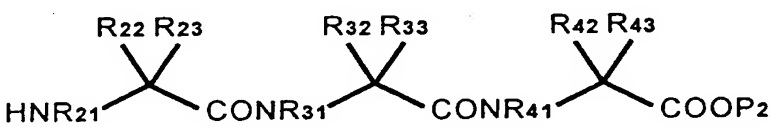
[9] A method for producing the compound of [1], which comprises the steps of:
reacting a compound represented by formula (2)



(2)

(wherein, n is same as that defined in formula (1); Hal denotes a halogen atom selected from a chlorine atom, bromine atom, or iodine atom, or an allyl or alkylsulfoxy group useful for a free group; P_2 denotes a protection group for an amino group);

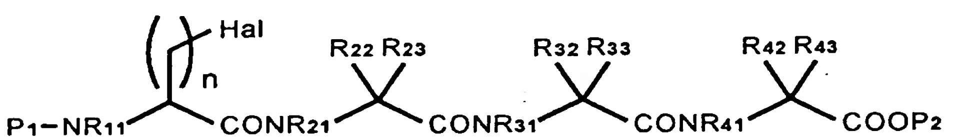
with a compound represented by formula (3).



(3)

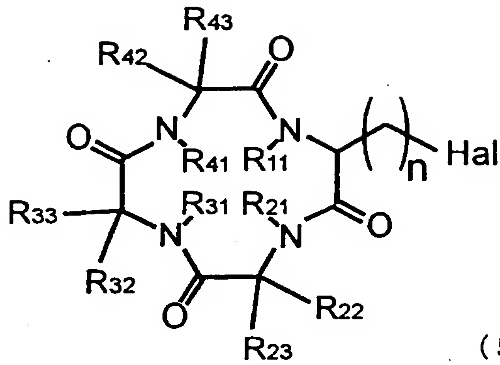
(wherein R_{11}, R_{21}, R_{22}, R_{23}, R_{31}, R_{32}, R_{33}, R_{41}, R_{42}, and R_{43} are same as defined in formula (1); P_2 denotes a protection group for a carboxyl group);

in the presence of a peptide-bonding agent to obtain a compound represented by formula (4)



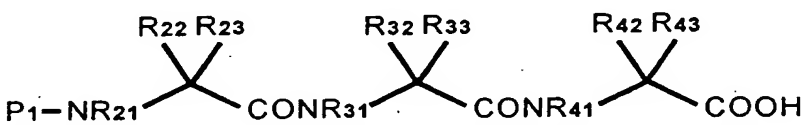
(4)

(wherein n, R_{11}, R_{21}, R_{22}, R_{23}, R_{31}, R_{32}, R_{33}, R_{41}, R_{42}, R_{43}, P_1, P_2, and Hal are the same as defined above); subjecting the compound represented by formula (4) to catalytic hydrogenation, acid treatment, or hydrolysis to remove P_1 and P_2; and then subjecting to cyclization in the presence of a peptide-bonding agent to obtain a compound represented by formula (5)


(5)

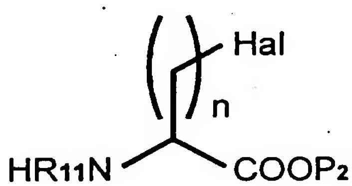
(wherein n, R_{11}, R_{21}, R_{22}, R_{23}, R_{31}, R_{32}, R_{33}, R_{41}, R_{42}, R_{43}, P_1, P_2, and Hal are the same as defined above);

or reacting a compound represented by formula (6)


(6)

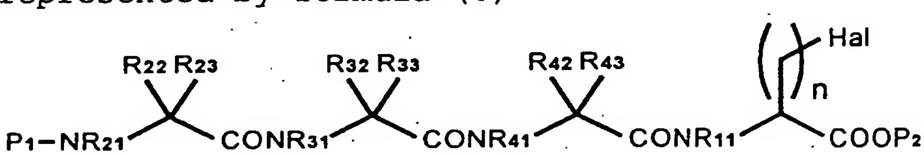
(wherein R_{21}, R_{22}, R_{23}, R_{31}, R_{32}, R_{33}, R_{41}, R_{42}, R_{43}, and P_1 are the same as defined above);

with a compound represented by formula (7)


(7)

(wherein n, R_{11}, P_2, and Hal are the same as defined above);

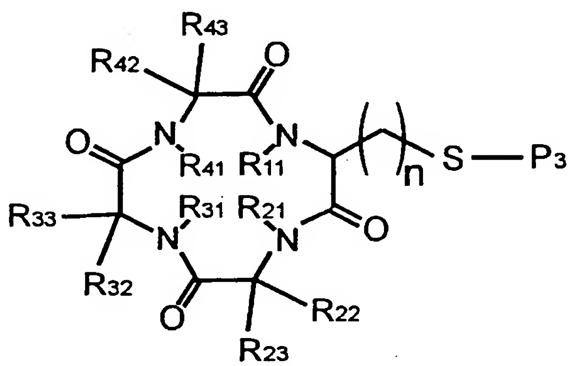
in the presence of a peptide-bonding agent to obtain a compound represented by formula (8)


(8)

(wherein n, R_{11}, R_{21}, R_{22}, R_{23}, R_{31}, R_{32}, R_{33}, R_{41}, R_{42}, R_{43}, P_1, P_2, and Hal are the same as defined above);

subjecting the compound represented by formula (8) to catalytic

hydrogenation, acid treatment, fluoride anion treatment, or hydrolysis to remove P_1 and P_2;
and then subjecting to cyclization in the presence of a peptide-bonding agent to obtain the compound represented by formula (5);
following, for both process, the steps of:
reacting the compound represented by formula (5) with a reagent comprising sulfur atoms to obtain a compound represented by formula (9)



(9)

(wherein n, R_{11}, R_{21}, R_{22}, R_{23}, R_{31}, R_{32}, R_{33}, R_{41}, R_{42}, and R_{43} are the same as defined above; P_3 denotes a protection group for sulfohydryl group); and then
treating the compound represented by formula (9) with an oxidizing agent as well as ammonia or another amine.

Hereinafter, modes for carrying out the present invention will be specifically described with reference to drawings.

The compounds of the present invention can be defined by the above-mentioned formula (1). Such compounds can be used as the HDAC inhibitors.

In the above-mentioned formula (1), R_{11}, R_{21}, R_{31}, and R_{41} may each independently be hydrogen or methyl. R_{22}, R_{23}, R_{32}, R_{33}, R_{42}, and R_{43} may each independently be hydrogen, a linear alkyl with one to six carbon atoms, or a non-aromatic cyclic alkyl group, in which the linear alkyl group with one to six carbon atoms and the non-aromatic cyclic alkyl group may bind with a non-aromatic cyclic alkyl group, or substituted or unsubstituted aromatic ring. Pairs of R_{21} and R_{22}, R_{22} and R_{23}, R_{31} and R_{32}, R_{32} and R_{33}, R_{41}

and R_{42}, and R_{42} and R_{43} can each independently be in an acyclic structure without binding, or may bind through a linear alkylene group with a one- to five-carbon main chain, a linear alkylene group with a one- to five-carbon main chain comprising a branched chain with one to six carbons, or a linear alkylene group with a one- to five-carbon main chain comprising a ring structure of one to six carbons. Since the cyclic tetrapeptide structure portion is thought to function as a cap to seal a pocket of HDAC, this cap structure can be arbitrarily selected from the above-mentioned linear alkyl with one to six carbon atoms, aromatic cyclic alkyl, and aromatic groups that can substitute for them.

Furthermore, hydrogen can be used as X in formula (1) to directly form a thiol group with a neighboring sulfur atom that exhibits HDAC inhibition activity. However, if the thiol group formed by using a hydrogen as X is exposed, the resulting compound becomes unstable *in vivo*. Therefore, if X is a hydrogen, the present compounds are preferably combined with a means for their stable delivery to a desired site, such as a drug delivery system. In order to enhance the stability of thiol groups comprising HDAC inhibition activity, it is preferable that X is a substituent group which is metabolized *in vivo* and harmless in the living body. This kind of substituent group is preferably a group comprising a sulfur atom capable of forming a disulfide bond with the sulfur atom next to the X, and can be a group that itself shows some efficacy, and can also be a group that functions simply as a protective group. Such a substituent group comprising a sulfur atom can be:

a structure identical to that shown to the left of X; an alkyl group or aryl group in any structure comprising a sulfur atom capable of binding via a disulfide bond with the sulfur atom in the above-mentioned formula (1); or a sulfur atom binding with the sulfur atom bonded to the terminus of the above-mentioned R_{22}, R_{23}, R_{32}, R_{33}, R_{42}, or R_{43} and located to the left of X via an intra-molecular disulfide bond. In this case, if the substituent group has the same structure as that to the left of X, resulting

in a dimer structure, the disulfide bond is cut by *in vivo* metabolism to isolate an HDAC inhibitor comprising the activity of two molecules. Furthermore, any alkyl or aryl comprising a sulfur atom may have further substituted groups, or may be a structure capable of exhibiting an effect identical to or different from that of the HDAC inhibitor.

Examples of the SS-hybrid X atom groups of the present invention are alkylmercaptans such as methylmercaptan, benzylmercaptan and cyclohexylmercaptan, and aromatic mercaptans such as thiophenol and mercaptopyridine, as well as alkylmercaptans and allylmercaptans in which a portion of the atom groups in the structure of natural physiologically active substances, such as 5-azadeoxycytidine and retinoic acid, are substituted with thiol groups. Preferable examples are methylmercaptan, ethylmercaptan, mercaptoethanol, cysteamine, cysteine, thiophenol, 2-mercaptopyridine, 4-mercaptopyridine, 5′-mercapto-5-azadoxycytidine, 3′-mercapto-5-azadeoxycytidine, and thioretinol.

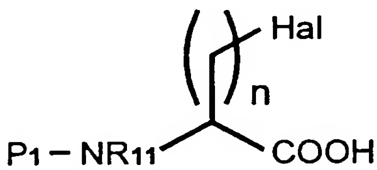
Furthermore, in the present invention, the ring n in formula (1) is not limited as long as it has HDAC inhibition activity and, for example, n is preferably 4 to 7, more preferably 5. The carbon chain comprising n carbon atoms, from the cyclic tetrapeptide structure to the sulfur atom, is supposed to enter the active HDAC pocket, and inhibit HDAC by contacting the active thiol group at the carbon chain end with the zinc molecule in the HDAC pocket.

Typical examples of the compounds of the present invention are shown in Figs. 1 to 3, but are not limited to these compounds.

Hereinafter, methods of producing the compounds of the present invention will be described. The compounds of this embodiment can be produced from 2-amino-n-haloalkanoic acid, as shown below. Since R_{11}, R_{21}, R_{22}, R_{23}, R_{31}, R_{32}, R_{33}, R_{41}, R_{42}, R_{43}, and n are defined according to the above descriptions, their descriptions are omitted.

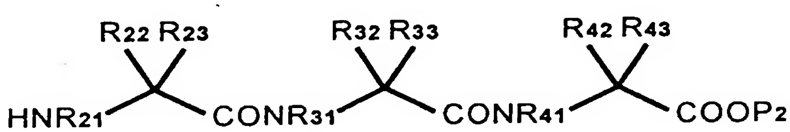
The first embodiment of methods of production for the

compounds of the present invention is a method that uses as raw material a compound of formula (2), in which protection group P_1 is linked to the amino group of 2-amino-n-haloalkanoic acid. Specifically, a compound defined by the following formula (2)



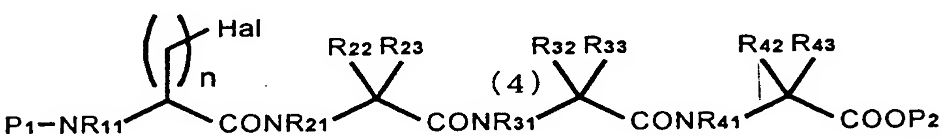
(2)

is reacted with a compound defined by the following formula (3)



(3)

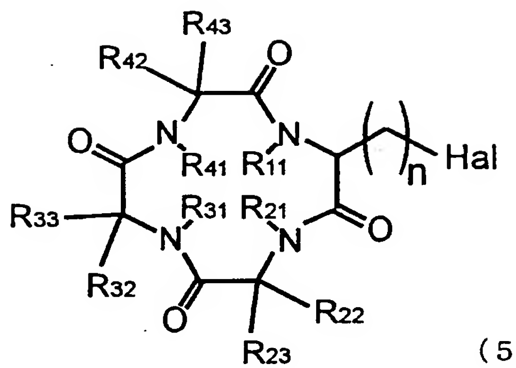
in the presence of a peptide-bonding agent to obtain a compound defined by the following formula (4).



(4)

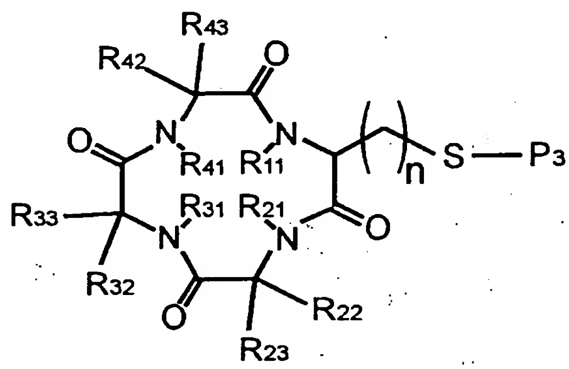
In the above-described formulae, Hal can be a halogen atom selected from any one of a chlorine atom, a bromine atom, or an iodine atom, or an allyl or alkylsulfoxy group that can also be a leaving group. P_2 is a protection group for an amino group.

Next, the compound defined by the above-mentioned formula (4) is subjected to catalytic hydrogenation, acid treatment, or hydrolysis for removing P_1 and P_2, and then to cyclization in the presence of a peptide-bonding agent, to obtain a compound defined by formula (5):



(5)

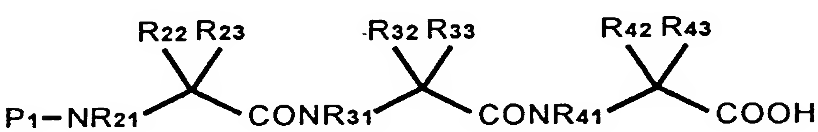
Next, the compound defined by formula (5) is reacted with a reagent comprising a sulfur atom to obtain a compound defined by formula (9):



(9)

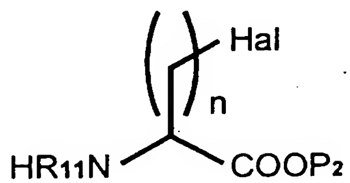
The compound defined by formula (9) is then treated with an oxidizing agent as well as ammonia or another amine to obtain a (dimer- or hybrid-type) prodrug compound comprising a disulfide bond. In formula (9), P_3 denotes a protection group for the sulfohydryl group. Treatment with a reducing agent or an enzyme capable of digesting disulfide bonds may be carried out to isolate active thiol-type compounds.

The second embodiment of the production methods of the present invention is a production method that uses as raw material a compound defined by the following formula (7), in which a protection group P_2 is linked to the carboxyl group of 2-amino-n-haloalkanoic acid. Specifically, a compound defined by formula (6)



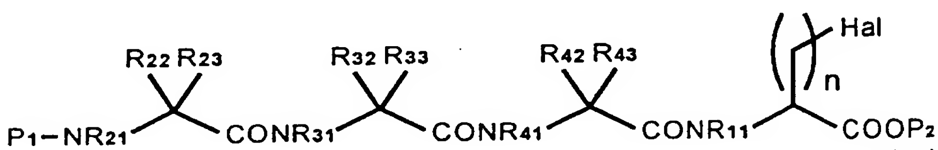
(6)

is reacted with a compound defined by formula (7)



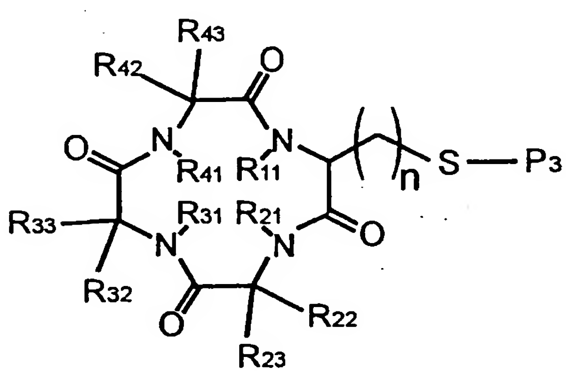
(7)

in the presence of peptide-bonding agent, to obtain a compound defined by the following formula (8):



(8)

The compound defined by formula (8) is then subjected to catalytic hydrogenation, acid treatment, fluoride anion treatment, or hydrolysis to remove P_1 and P_2, and then subjected to cyclization in the presence of a peptide-bonding agent, to obtain a compound defined by formula (5). Next, the compound defined by formula (5) is reacted with a sulfur-atom-comprising reagent to obtain a compound defined by formula (9):



(9)

The compound defined by formula (9) is then treated with an oxidizing agent as well as ammonia or another amine to obtain a (dimer- or hybrid-type) prodrug compound comprising a disulfide bond. As for the above-mentioned first embodiment, active thiol-type compounds may be isolated by treatment with a reducing agent or an enzyme capable of digesting disulfide bonds.

HDAC-inhibiting compounds are known to induce differentiation of cancer cells, leukemia cells, and neural

cells, to induce apoptosis, and suppress cancer cell metastasis (Yoshida, M., Nomura, S., and Beppu, T. Effects of trichostatins on differentiation of murine erythroleukemia cells. Cancer Res. 47: 3688-3691, 1987; Hoshikawa, Y., Kijima, M., Yoshida, M., and Beppu, T. Expression of differentiation-related markers in teratocarcinoma cells via histone hyperacetylation by trichostatin A. Agric. Biol. Chem. 55: 1491-1495, 1991; Minucci, S., Horn, V., Bhattacharyya, N., Russanova, V., Ogryzko, V. V., Gabriele, L., Howard, B. H., and Ozato, K. A histone deacetylase inhibitor potentiates retinoid receptor action in embryonal carcinoma cells. Proc. Natl. Acad. Sci. USA 94: 11295-11300, 1997; Inokoshi, J., Katagiri, M., Arima, S., Tanaka, H., Hayashi, M., Kim, Y. B., Furumai, R., Yoshida, M., Horinouchi, S., and Omura, S. (1999). Neuronal differentiation of Neuro 2a cells by inhibitors of cell progression, trichostatin A and butyrolactone I. Biochem. Biophys. Res. Commun. 256, 372-376; Wang, J., Saunthararajah, Y., Redner, R. L., and Liu, J. M. Inhibitors of histone deacetylase relieve ETO-mediated repression and induce differentiation of AML1-ETO leukemia cells. Cancer Res. 59: 2766-2769, 1999; Munster, P. N., Troso-Sandoval, T., Rosen, N., Rifkind, R., Marks, P. A., and Richon, V. M. The histone deacetylase inhibitor suberoylanilide hydroxamic acid induces differentiation of human breast cancer cells. Cancer Res. 61: 8492-8497, 2001; Ferrara, F. F., Fazi, F., Bianchini, A., Padula, F., Gelmetti, V., Minucci, S., Mancini, M., Pelicci, P. G., Lo Coco, F., and Nervi, C. Histone deacetylase-targeted treatment restores retinoic acid signaling and differentiation in acute myeloid leukemia. Cancer Res. 61: 2-7, 2001; Gottlicher, M., Minucci, S., Zhu, P., Kramer, O. H., Schimpf, A., Giavara, S., Sleeman, J. P., Lo Coco, F., Nervi, C., Pelicci, P. G., and Heinzel, T. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. EMBO J. 20: 6969-6978, 2001). Accordingly, the compounds of the present invention can be utilized as apoptosis-inducing agents, differentiation-inducing agents, and cancer-metastasis-suppressing agents.

Also, the compounds inhibiting HDAC are expected to inhibit angiogenesis (Kim, M. S., Kwon, H. J., Lee, Y. M., Baek, J. H., Jang, J. E., Lee, S. W., Moon, E. J., Kim, H. S., Lee, S. K., Chung, H. Y., Kim, C. W., and Kim, K. W. (2001). Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes. Nature Med. 7, 437-443; Kwon, H. J., Kim, M. S., Kim, M. J., Nakajima, H., and Kim, K. W. (2002). Histone deacetylase inhibitor FK228 inhibits tumor angiogenesis. Int. J. Cancer 97, 290-296). Thus, the compounds of the present invention can be also utilized as angiogenesis inhibitors.

Among various HDACs, the compounds of the present invention exhibit a strong inhibitive activity specific to HDAC1 and HDAC4. Therefore, the compounds of the present invention are useful as pharmaceutical agents for treating or preventing diseases caused by HDAC1 and HDAC4. Examples of such diseases besides cancer include autoimmune diseases, skin diseases, and infectious diseases associated with HDAC1 and HDAC4. Furthermore, the compounds of the present invention may be applied not only to pharmaceutical agents for treating or preventing the above-mentioned diseases, but also to gene therapy adjuvants or accelerating agents that improve the efficiency of vector introduction, promote the expression of introduced genes, and the like.

The compounds of the present invention may also be used in combination with retinoic acids and DNA methylation inhibitors. The invention also provides such concomitant agents.

When formulating the compounds of the present invention, fillers, extenders, binders, moisturizing agents, disintegrators, surfactants, diluents such as lubricants, and vehicles may be used as necessary. Furthermore, coloring agents, preservatives, aromatics, flavors, sweeteners, and other pharmaceuticals may be added to the pharmaceutical formulations. The form of each type of pharmaceutical formulation may be selected in line with its therapeutic or preventative purpose. The form may be, for example, a tablet, pill, powder, solution, suspension, emulsion, granule, capsule, injection, and suppository.

Examples of additives to be added to tablets and capsules include binders such as gelatin, corn starch, tragacanth gum, and acacia; vehicles such as crystalline cellulose; swelling agents such as corn starch, gelatin, and alginic acid; lubricants such as magnesium stearate; sweeteners such as sucrose, lactose, and saccharine; and aromatics such as peppermint, Gaultheria adenothrix oil, and cherry. In the case where the unit dosage form is a capsule, a liquid carrier such as oil or fat can be added in addition to the above-mentioned materials.

As an aqueous solution for injection, an isotonic solution of, for example, D-sorbitol, D-mannose, D-mannitol, or sodium chloride comprising saline, glucose, and other adjuvants may also be used as necessary in combination with a proper dissolution-assisting agent, such as an alcohol, specifically, ethanol, a polyalcohol such as propylene glycol and polyethylene glycol, or a nonionic surfactant such as polysorbate 80™ and HCO-50.

Examples of an oleaginous solution are sesame oil and soybean oil, which can be used, as necessary, in combination with a dissolution-assisting agent such as benzyl benzoate and benzyl alcohol. Furthermore, mixing with a buffer such as phosphate buffer solution or sodium acetate buffer solution; a soothing agent such as procaine hydrochloride; a stabilizer such as benzyl alcohol and phenol; or an antioxidant is also acceptable. The formulated injection is generally filled into suitable ampules.

Formulations may be administered to patients orally or parenterally. Examples of a parenteral dosage form, include injection as well as transnasal, transpulmonal, and transdermal administration. Systemic or local administration can be carried out using an injection dosage form, such as intravenous injection, intramuscular injection, intraperitoneal injection, and subcutaneous injection. Furthermore, intranasal, transbronchial, intramuscular, subcutaneous, or oral administration may also be carried out by methods known to those

skilled in the art.

For parenteral administration, the unit dosage of the compounds of the present invention depends on the subjects to be administrated, the target organs, symptoms, and the manner of administration. For example, it is preferable that injections are administered intravenously to adults (60 kg body weight) at a dosage of about 0.01 to 30 mg per day, preferably about 0.1 to 20 mg per day, and more preferably about 0.1 to 10 mg per day. When administering to other kind of animals, dosage can be converted per 60 kg body weight, or per unit of body surface area.

For oral administration, the unit dosage of the compounds of the present invention depends on the subjects to be administrated, the target organs, symptoms, and manner of administration, and is preferably, for example, about 100 μg to 20 mg per day for an adult (60 kg body weight).

<u>Brief Descriptions of the Drawings</u>

Fig. 1 shows a list of the structure of LDLD or LDLL isomers of SCOP with five carbon chains until the active thiol group.

Fig. 2 shows the structures of SCOPs with four to seven carbon chains until the active thiol group. Note that SCOP 152 (C5) is the same here as in Fig. 1.

Fig. 3 shows the structures of homodimer-type SCOPs. The SCOP numbers are twice the number of monomers.

Fig. 4 shows the structures of hybrid-type SCOPs, obtained by bonding a variety of compounds to SCOP 152.

Fig. 5 shows the steric conformation of natural Cyl-1 and Cyl-2.

Fig. 6 shows photographs of the results of measuring intracellular histone acetylation level by Western blot analysis using anti-acetylated lysine antibodies.

Fig. 7 shows the results of evaluating the stability of SCOP 152, SCOP 304, and SCOP 402 in serum.

Fig. 8 shows photographs of the results of evaluating the

stability of SCOP 152, SCOP 304, and SCOP 402 on a cellular level.

Best Mode for Carrying Out the Invention

Hereinafter, Figs. 1 and 2 show the entire flow of the process of synthesizing the compounds shown in these examples. Each synthesis step is described in detail below, with H-L-Ab7-OH as the starting material. In the following, 2-amino-7-bromoheptanoic acid is abbreviated to "Ab7"; 2-amino-7-acetyltioheptanoic acid is "Am7(Ac)"; 2-amino-7-mercaptoheptanioc acid is "Am7"; sulfide of 2-amino-7-mercaptoheptanioc acid is "Am7(-)"; 2-amino-8.9-dimercapto(S^9-2'-nitro-N,N'-dimethyl benzamide) is "Am7(E11)"; 2-amino-8,9-dimercapto-11-hydroxyundecanoic acid is "Am7(SMEt)"; 2-amino-8.9-dimercapto(S^9-2'-pyridyl) nonanoic acid is "Am7(S2Py)"; 2-amino-8.9-dimercapto(S^9-4'-pyridyl) nonanoic acid is "Am7(S4Py)"; and 2-amino-8,9-dimercaptodecanoic acid is "Am7(SMe)". In addition, sulfur-containing cyclic peptides, which are synthesized compounds, are abbreviated to "SCOP".

[Example 1] Synthesis of Boc-L-Ab7-OH

H-L-Ab7-OH (7.3 g, 32.4 mmol) was dissolved in water:dioxane = 1:1 solution (30 ml, v/v). While cooling on ice, $(Boc)_2O$ (7.68 g, 35.6 mmol) and triethylamine (6.72 ml, 48.6 mmol) were added to the mixture, which was then stirred for five hours. After the reaction solution was evaporated, the residue was washed with ether. The aqueous phase was acidified using citric acid and reverse-extracted with ethyl acetate. The extract was dried over $MgSO_4$ and ethyl acetate was then removed by evaporation. After vacuum drying, the oily title compound (10.4 g, 32.4 mmol, 100% yield) was obtained.

[Example 2] Synthesis of Boc-L-Ab7-NHMe

While cooling on ice, triethylamine (0.17 ml, 1.2 mmol) and DCC (247 mg, 1.2 mmol) were added to 3 ml of DMF containing Boc-L-Ab7-OH (326 mg, 1.0 mmol), monomethylamine hydrochloride (81

mg, 1.2 mmol), and HOBt·H_2O (184 mg, 1.2 mmol). After stirring for 15 hours, the DMF was removed by evaporation. The residue was dissolved in ethyl acetate and successively washed with an aqueous 10% citric acid solution, an aqueous 4% sodium bicarbonate solution, and saturated saline. This was then dried over $MgSO_4$ and concentrated. The resulting oily substance was purified by flash silica gel chromatography (3.6x 15 cm, chloroform) and solidified by adding ether/petroleum ether (1:10) to obtain the white powder of the title compound (250 mg, 0.74 mmol, 74% yield). TLC: Rf= 0.58 ($CHCl_3$/MeOH = 9/1).

[Example 3] Synthesis of Boc-L-Am7(Ac)-NHMe

Potassium thioacetate (64 mg, 0.56 mmol) was added to DMF (2 ml) containing Boc-Ab7-NHMe (125 mg, 0.37 mmol) and reacted for 3 hours. After DMF was removed by evaporation, the residue was dissolved in ethyl acetate and successively washed with an aqueous 10% citric acid solution and saturated saline. The product was dried over $MgSO_4$, concentrated, and solidified by adding ether/petroleum ether (1:10) to obtain the white powder of the title compound (120mg, 0.36 mmol, 97% yield). TLC: Rf= 0.57 ($CHCl_3$/MeOH = 9/1).

[Example 4] Synthesis of Boc-L-Am7(-)-NHMe SS-dimer

Methanolic ammonia (20 eq.) was added to DMF (0.5 ml) containing Boc-Am7(Ac)-NHMe (60 mg, 0.18 mmol) and stirred for 24 hours. After concentrating the reaction solution, the SS-dimer produced was purified by flash silica gel chromatography (1.5x 30 cm, 1% methanol/chloroform) to obtain the white powder of the title compound (43 mg, 0.11 mmol, 61% yield). HPLC retention time: 8.5 min; HRMS (FAB, dithiodiethanol), 579.3293 [M+H], $C_{26}H_{51}O_6N_4S_2$ (579.3250).

[Example 5] Synthesis of Boc-L-Am7(S4Py)-NHMe

4,4'-dithiodipyridine (79 mg, 0.36 mmol) and methanolic ammonia (20 eq.) were added to DMF (0.5 ml) containing Boc-Am7(Ac)-NHMe (60 mg, 0.18 mmol), and stirred for 5 hours. After

the reaction solution was concentrated, the resulting oily product was purified by flash silica gel chromatography (1.5x 30 cm, chloroform) and freeze-dried to obtain the title compound (43 mg, 0.11 mmol, 61% yield). HPLC retention time: 5.6 min; HRMS (FAB, dithiodiethanol), 400.1766 [M+H], $C_{18}H_{29}O_3N_3S_2$ (400.1729).

[Example 6] Synthesis of Boc-L-Ab7-OBzl

Boc-L-Ab7-OH (4.05 g, 12.5 mmol) was dissolved in DCM (20 ml). While cooling on ice, benzyl alcohol (1.55 ml, 15.0 mmol), 4-dimethylaminopyridine (153 mg, 1.25 mmol), and DCC (3.09 g, 15.0 mmol) were added to the mixture, which was then stirred for 8 hours. After the reaction solution was evaporated, the residue was dissolved in ethyl acetate and successively washed with an aqueous 10% citric acid solution, an aqueous 4% sodium bicarbonate solution, and saturated saline. This was then dried over $MgSO_4$, concentrated, and the resulting oily substance was purified by flash silica gel chromatography (5x 20 cm, 20% ethyl acetate/hexane) to obtain the oily title compound (4.29 g, 10.4 mmol, 83% yield). TLC: Rf= 0.49 (ethyl acetate/hexane = 1/4).

[Example 7] Synthesis of Boc-L-Ile-L-Pro-OBzl

While cooling on ice, Boc-L-Pro-OH (1.08 g, 5.0 mmol) and benzyl bromide (0.893 ml, 75 mmol) were reacted in DMF (10 ml) in the presence of triethylamine (10.5 ml, 75 mmol) to obtain Boc-L-Pro-OBzl as an oily product. This product was reacted under ice-cooling with 2 N HCl/dioxane (5 eq.) for three hours to obtain H-L-Pro-OBzl·HCl.

While cooling on ice, DCC (1.24 g, 6.0 mmol) and triethylamine (0.70 ml, 4.0 mmol) were added to DMF (10 ml) containing Boc-L-Ile-OH·1/2 H_2O (1.39 g, 6.0 mmol), H-D-Pro-OBzl·HCl (956 mg, 4.0 mmol), and HOBt·H_2O (613 mg, 4.0 mmol). After stirring for 8 hours, DMF was removed by evaporation, the residue was dissolved in ethyl acetate, and then successively washed with an aqueous 10% citric acid solution, an aqueous 4% sodium bicarbonate solution, and saturated saline. After drying

over $MgSO_4$ and concentrating, the resulting oily substance was purified by flash silica gel chromatography (4x 30 cm, 1% methanol/chloroform) to obtain the oily title compound (1.63 g, 3.38 mmol, 85% yield). TLC: Rf= ($CHCl_3$/MeOH = 9/1)

[Example 8] Synthesis of Boc-D-Tyr(Me)-L-Ile-L-Pro-OBzl

Boc-L-Ile-L-Pro-OBzl (1.63 g, 3.38 mmol) was dissolved in TFA (5 ml) and left with standing on ice for 30 minutes. On completion of the reaction, TFA was removed by evaporation, and the residue was vacuum-dried to obtain H-L-Ile-L-Pro-OBzl·TFA. The compound was dissolved in DMF (8 ml), and Boc-D-Tyr(Me)-OH (1.50 g, 5.07 mmol) was then added. HBTU (1.92 g, 5.07 mmol), HOBt·H_2O (518 mg, 3.38 mmol), and triethylamine (2.37 ml, 16.9 mmol) were further added and stirred for three hours under ice-cooling. The reaction solution was concentrated, dissolved in ethyl acetate, and successively washed with an aqueous 10% citric acid solution, an aqueous 4% sodium bicarbonate solution, and saturated saline. The product was dried over $MgSO_4$ and concentrated, and the resulting foam substance was purified by flash silica gel chromatography (4x 30 cm, 1% methanol/chloroform) to obtain the foam title compound (1.44 g, 2.42 mmol, 72% yield). TLC: Rf= ($CHCl_3$/MeOH = 9/1).

[Example 9] Synthesis of Boc-D-Tyr(Me)-L-Ile-L-Pro-L-Ab7-OBzl

Boc-D-Tyr(Me)-L-Ile-L-Pro-OBzl (1.44 g, 2.42 mmol) was dissolved in methanol (12 ml) and subjected to catalytic hydrogenation in the presence of the 5% Pd-C (150 mg). After five hours, the catalyst Pd-C was filtered and the reaction solution was removed by evaporation to obtain Boc-D-Tyr(Me)-L-Ile-L-Pro-OH·TFA.

Boc-L-Ab7-OBzl (1.29 g, 3.12 mmol) was dissolved in TFA (10 ml) and left with standing on ice for 30 minutes. On completion of the reaction, TFA was removed by evaporation, and the residue was vacuum-dried to obtain H-L-Ab7-OH·TFA. The compound was dissolved in DMF (16 ml), and Boc-D-Tyr(Me)-L-Ile-L-Pro-OH (1.21 g, 2.40 mmol) was then added. HBTU (1.18 g, 3.12 mmol), HOBt·H_2O

(368 mg, 2.40 mmol), and triethylamine (1.34 ml, 9.6 mmol) were further added and stirred for 3 hours under ice-cooling. The reaction solution was concentrated, dissolved in ethyl acetate, and successively washed with 10% citric acid, an aqueous 4% sodium bicarbonate solution, and saturated saline. The product was dried over $MgSO_4$ and concentrated, and the resulting foam substance was purified by flash silica gel chromatography (4x 30 cm, 2% methanol/chloroform) to obtain the foam title compound (1.20 g, 1.47 mmol, 61% yield). TLC: Rf= ($CHCl_3$/MeOH = 9/1).

[Example 10] Synthesis of H-D-Tyr(Me)-L-Ile-L-Pro-L-Ab7-OH·TFA

Boc-D-Tyr(Me)-L-Ile-L-Pro-L-Ab7-OBzl (1.20 g, 1.47 mmol) was dissolved in methanol (7.5 ml) and subjected to catalytic hydrogenation in the presence of the catalyst Pd-C (130 mg). After 5 hours, the catalyst Pd-C was filtered and the reaction solution was removed by evaporation to obtain Boc-D-Tyr(Me)-L-Ile-L-Pro-L-Ab7-OH. This compound was dissolved in TFA (5 ml) and left standing on ice for 30 minutes. After the reaction solution was removed by evaporation, ether/petroleum ether (1:10) was added to the residue for solidification. This was then vacuum-dried to obtain the title compound (770 mg, 1.02 mmol, 69% yield).

[Example 11] Synthesis of cyclo(-L-Ab7-D-Tyr(Me)-L-Ile-L-Pro-)

H-D-Tyr(Me)-L-Ile-L-Pro-L-Ab7-OH·TFA (770 mg, 1.02 mmol), HATU (388 mg, 1.53 mmol), and DIEA (0.71 ml) were divided into five aliquots. DMF (1000 ml) was added to each aliquot every 30 minutes, and cyclization reaction was carried out. After 2 hours, the solvent was removed by evaporation. The residue was dissolved in ethyl acetate, successively washed with an aqueous 10% citric acid solution, 4% $NaHCO_3$, and saturated saline, and then dried with $MgSO_4$. Ethyl acetate was removed by evaporation, and the remaining oily substance was purified by flash silica gel chromatography (4x 30 cm, 2% methanol/chloroform) to obtain a foam substance 130 mg (21%). HPLC retention time: 8.20 min; FAB-MS (dithiodiethanol), 593 [M+H], (593.2).

[Example 12] Synthesis of cyclo(-L-Am7(Ac)-D-Tyr(Me)-L-Ile-L-Pro-).

Potassium thioacetate (9.59 mg, 0.084 mmol) was added to DMF (0.5 ml) containing cyclo(-L-Ab7-D-Tyr(Me)-L-Ile-L-Pro-) (25 mg, 0.042 mmol) and reacted for 3 hours. DMF was removed by evaporation. The residue was dissolved in ethyl acetate and then successively washed with aqueous 10% citric acid solution, 4% $NaHCO_3$, and saturated saline. The thioester produced similarly after the cyclization reaction was isolated and purified to obtain 19 mg (76%) of oily product. HPLC retention time: 8.20 min; FAB-MS (dithiodiethanol), 589 [M+H], (589.3).

[Example 13] Synthesis of cyclo(-L-Am7(-)-D-Tyr(Me)-L-Ile-L-Pro-) (SS-dimer: SCOP 296)

Cyclo(-L-Am7(Ac)-D-Tyr(Me)-L-Ile-L-Pro-) (19 mg, 0.0322 mmol) was dissolved in hot DMF (2 ml) and reacted with methanolic ammonia (10 eq.) to remove the acetyl groups. After the solvent was removed by evaporation, the residue was dissolved in DMF (2 ml), and 1 M I_2 (ethanol) (0.04 ml) was added to the solution for oxidization. The produced SS-dimer was purified through a Sephadex LH-20 (DMF) column and then mixed with water to obtain a white powder. The yield was 7.4 mg (42%). HPLC retention time: 14.1 min; HRMS (FAB, dithiodiethanol), 1091.5648 [M+H], $C_{56}H_{83}O_{10}N_8S_2$ (1091.5674).

[Example 14] Synthesis of Boc-L-Ile-DL-Pip-OBzl

Boc-DL-Pip-OH (2.29 g, 10 mmol) and benzyl bromide (1.79 ml, 15 mmol) were reacted in DMF (20 ml) in the presence of triethylamine (2.1 ml, 15 mmol) to obtain Boc-DL-Pip-OBzl as an oily product. This product was reacted with 2 N HCl/dioxane (5 eq.) for 3 hours to obtain H-DL-Pip-OBzl·HCl.

While cooling on ice, DCC (2.20 g, 10.7 mmol) and triethylamine (1.25 ml, 8.9 mmol) were added to DMF (20 ml) containing Boc-L-Ile-OH·1/2 H_2O (2.47 g, 10.7 mmol), H-D-Pro-OBzl·HCl (2.28 g, 8.9 mmol), and HOBt·H_2O (1.36 mg, 8.9 mmol).

After stirring for 8 hours, DMF was removed by evaporation, the residue was dissolved in ethyl acetate, and then successively washed with an aqueous 10% citric acid solution, an aqueous 4% sodium bicarbonate solution, and saturated saline. After drying over $MgSO_4$ and concentrating, the resulting oily substance was purified by flash silica gel chromatography (4x 30 cm, 1% methanol/chloroform) to obtain the oily title diastereomer mixture (3.33 g, 7.70 mmol, 87% yield). TLC: Rf= ($CHCl_3$/MeOH = 9/1)

[Example 15] Synthesis of Boc-D-Tyr(Me)-L-Ile-DL-Pip-OBzl

Boc-L-Ile-DL-Pip-OBzl (3.33 g, 7.70 mmol) was dissolved in TFA (10 ml) and left with standing on ice for 30 minutes. On completion of the reaction, TFA was removed by evaporation, and the residue was vacuum-dried to obtain H-L-Ile-DL-Pip-OBzl·TFA. The compound was dissolved in DMF (16 ml), and Boc-D-Tyr(Me)-OH (3.41 g, 11.6 mmol) was then added. HBTU (4.38 g, 11.6 mmol), HOBt·H_2O (1.18 g, 7.70 mmol), and triethylamine (7.01 ml, 50.1 mmol) were further added and stirred for 3 hours under ice-cooling. The reaction solution was concentrated, dissolved in ethyl acetate, and successively washed with an aqueous 10% citric acid solution, an aqueous 4% sodium bicarbonate solution, and saturated saline. The product was dried over $MgSO_4$ and concentrated, and the resulting foam substance was purified by flash silica gel chromatography (4x 30 cm, 1% methanol/chloroform) to obtain the foam title diastereomer mixture (3.46 g, 5.67 mmol, 74% yield). TLC: Rf= ($CHCl_3$/MeOH = 9/1).

[Example 16] Synthesis of Boc-D-Tyr(Me)-L-Ile-DL-Pip-L-Ab7-OBzl

Boc-D-Tyr(Me)-L-Ile-DL-Pip-OBzl (3.46 g, 7.37 mmol) was dissolved in methanol (30 ml) and subjected to catalytic hydrogenation in the presence of the 5% Pd-C (230 mg). After 8 hours, the catalyst Pd-C was filtered and the reaction solution was evaporated to obtain Boc-D-Tyr(Me)-L-Ile-DL-Pip-OH.

Boc-L-Ab7-OBzl (3.05 g, 3.12 mmol) was dissolved in TFA (5

ml) and left with standing on ice for 30 minutes. On completion of the reaction, TFA was removed by evaporation, and the residue was vacuum-dried to obtain H-L-Ab7-OBzl·TFA. The compound was dissolved in DMF (16 ml), and Boc-D-Tyr(Me)-L-Ile-DL-Pip-OH (2.80 g, 5.39 mmol) was then added. HBTU (2.66 g, 7.01 mmol), HOBt·H_2O (825 mg, 5.39 mmol), and triethylamine (3.02 ml, 21.6 mmol) were further added and stirred for 3 hours under ice-cooling. The reaction solution was concentrated, dissolved in ethyl acetate, and successively washed with an aqueous 10% citric acid solution, an aqueous 4% sodium bicarbonate solution, and saturated saline. The product was dried over $MgSO_4$ and concentrated, and the resulting foam substance was purified by flash silica gel chromatography (4x 30 cm, 2% methanol/chloroform) to obtain the foam title diastereomer mixture (4.07 g, 4.91 mmol, 91% yield). TLC: Rf= ($CHCl_3$/MeOH = 9/1).

[Example 17] Synthesis of H-D-Tyr(Me)-L-Ile-DL-Pip-L-Ab7-OH·TFA

Boc-D-Tyr(Me)-L-Ile-L-Pro-DL-Pip-OBzl (4.07 g, 4.91 mmol) was dissolved in methanol (10 ml) and subjected to catalytic hydrogenation in the presence of the catalyst Pd-C (300 mg). After 8 hours, the catalyst Pd-C was filtered and the reaction solution was removed by evaporation to obtain Boc-D-Tyr(Me)-L-Ile-DL-Pip-OH. This compound was dissolved in TFA (10 ml) and left standing on ice for 30 minutes. After the reaction solution was concentrated by evaporation, ether/petroleum ether (1:10) was added to the residue for solidification. This was then vacuum-dried to obtain the title diastereomer mixture (2.60 g, 3.51 mmol, 72% yield).

[Example 18] Synthesis of cyclo(-L-Ab7-D-Tyr(Me)-L-Ile-L-Pip-) and cyclo(-L-Ab7-D-Tyr(Me)-L-Ile-D-Pip-)

A linear tetrapeptide, H-D-Tyr(Me)-L-Ile-DL-Pip-L-Ab7-OH (1.28 g, 2.0 mmol), HATU (1.14 g, 3.0 mmol), and DIEA (1.0 ml) were divided into five aliquots. DMF (1000 ml) was added to each aliquot every 30 minutes, and cyclization reaction was carried

out. After 2 hours, the reaction solution was concentrated, dissolved in ethyl acetate, and successively washed with an aqueous 10% citric acid solution, an aqueous 4% sodium bicarbonate solution, and saturated saline. After drying up over $MgSO_4$ and concentrating, the resulting foam substance was purified by flash silica gel chromatography (4x 30 cm, 2% methanol/chloroform) to obtain a foam cyclo(-L-Ab7-D-Tyr(Me)-L-Ile-L-Pip-) (372 mg, 61%; HPLC retention time: 8.94 min; FAB-MS (dithiodiethanol), 607 [M+H], (607.2)) and a foam cyclo(-L-Am7(-)-D-Tyr(Me)-L-Ile-D-Pip-) (238 mg, 39%; HPLC retention time: 10.5 min; FAB-MS (dithiodiethanol), 607 [M+H], (607.2)).

[Example 19] Synthesis of cyclo(-L-Am7(Ac)-D-Tyr(Me)-L-Ile-L-Pip-)

Potassium thioacetate (69 mg, 0.315 mmol) was added to DMF (1 ml) containing cyclo(-L-Ab7-D-Tyr(Me)-L-Ile-L-Pip-) (130 mg, 0.21 mmol) and reacted for 3 hours. The reaction solution was concentrated by evaporation, dissolved in ethyl acetate, and then successively washed with aqueous 10% citric acid solution and saturated saline. The thioester produced similarly after the cyclization reaction was isolated and purified to obtain 109 mg (86%) of oily product. HPLC retention time: 8.94 min; FAB-MS (dithiodiethanol), 603 [M+H], (603.3).

[Example 20] Synthesis of cyclo(-L-Am7(-)-D-Tyr(Me)-L-Ile-L-Pip-) (SS-dimer: SCOP 298)

Methanol solution (0.5 ml) containing cyclo(-L-Am7(Ac)-D-Tyr(Me)-L-Ile-L-Pip-) (114 mg, 0.198 mmol) was reacted with methanolic ammonia (10 eq.) to remove the acetyl groups. After the solvent was removed by evaporation, the residue was dissolved in DMF (2 ml), and 1 M I_2 (ethanol) (0.25 ml) was added to the solution for oxidization. The produced SS-dimer was purified through a Sephadex LH-20 (DMF) column and then mixed with water to obtain a white powder. The yield was 82 mg (78%). HPLC retention time: 11.6 min; HRMS (FAB, dithiodiethanol), 1063.5391 [M+H], $C_{54}H_{79}O_{10}N_8S_2$ (1063.5361).

[Example 21] Synthesis of cyclo(-L-Am7(Ac)-D-Tyr(Me)-L-Ile-D-Pip-)

Potassium thioacetate (69 mg, 0.60 mmol) was added to DMF (0.5 ml) containing cyclo(-L-Ab7-D-Tyr(Me)-L-Ile-D-Pip-) (240 mg, 0.40 mmol), and this was reacted for 3 hours. The reaction solution was concentrated, dissolved in ethyl acetate, and successively washed with aqueous 10% citric acid solution, an aqueous 4% sodium bicarbonate solution, and saturated saline. After drying over $MgSO_4$ and concentrating, the resulting thioester was isolated and purified in the same manner as after the cyclization reaction to obtain an oily substance (160 mg) (66%). HPLC retention time: 10.5 min; FAB-MS (dithiodiethanol), 603 [M+H], (603.3).

[Example 22] Synthesis of cyclo(-L-Am7(-)-D-Tyr(Me)-L-Ile-D-Pip-) (SS-dimer: SCOP 300)

DMF (10 ml) containing cyclo(-L-Am7(Ac)-D-Tyr(Me)-L-Ile-D-Pip-) (160 mg, 0.27 mmol) was reacted with methanolic ammonia (10 eq.) to remove the acetyl groups. After the solvent was removed by evaporation, the residue was dissolved in DMF (2 ml), and 1 M I_2 (ethanol) (0.31 ml) was added to the solution for oxidization. The produced SS-dimer was purified through a Sephadex LH-20 (DMF) column and then mixed with water to obtain a white powder. The yield was 54 mg (36%). HPLC retention time: 13.4 min; HRMS (FAB, dithiodiethanol), 1119.5939 $[M+H]^+$, $C_{58}H_{87}O_{10}N_8S_2$ (1119.5986).

[Example 23] Synthesis of Boc-L-Ile-D-Pro-OBzl

While cooling on ice, Boc-D-Pro-OH (17.2 g, 80 mmol) and benzyl bromide (14.3 ml, 120 mmol) were reacted in DMF (160 ml) in the presence of triethylamine (16.8 ml, 120 mmol) to obtain Boc-D-Pro-OBzl as an oily product. This product was reacted with 2 N HCl/dioxane (5 eq.) for 3 hours to obtain H-D-Pro-OBzl·HCl.

While cooling on ice, DCC (8.3 g, 30 mmol) and triethylamine (3.5 ml, 25 mmol) were added to DMF (200 ml)

containing Boc-L-Ile-OH·1/2 H_2O (24.0 g, 100 mmol), H-D-Pro-OBzl·HCl (19.3 g, 80 mmol), and HOBt·H_2O (15.3 g, 100 mmol). After stirring for 8 hours, DMF was removed by evaporation, the residue was dissolved in ethyl acetate, and then successively washed with an aqueous 10% citric acid solution, an aqueous 4% sodium bicarbonate solution, and saturated saline. After drying over $MgSO_4$ and concentrating, the resulting oily substance was purified by flash silica gel chromatography (4x 30 cm, 1% methanol/chloroform) to obtain the oily title compound (21.5 g, 51 mmol, 72% yield). TLC: Rf= ($CHCl_3$/MeOH = 9/1)

[Example 24] Synthesis of Boc-D-Tyr(Me)-L-Ile-D-Pro-OBzl

Boc-L-Ile-D-Pro-OBzl (21.5 g, 51.4 mmol) was dissolved in TFA (50 ml) and left with standing on ice for one hour. On completion of the reaction, TFA was removed by evaporation, and the residue was vacuum-dried to obtain H-L-Ile-D-Pro-OBzl·TFA. The compound was dissolved in DMF (100 ml), and Boc-D-Tyr(Me)-OH (16.7 g, 56.5 mmol) was then added. HBTU (29.4 g, 77 mmol), HOBt·H_2O (7.87 g, 51 mmol), and triethylamine (25.2 ml, 180 mmol) were further added and stirred for 3 hours under ice-cooling. The reaction solution was concentrated, dissolved in ethyl acetate, and successively washed with an aqueous 10% citric acid solution, an aqueous 4% sodium bicarbonate solution, and saturated saline. The product was dried over $MgSO_4$ and concentrated, and the resulting foam substance was purified by flash silica gel chromatography (4x 30 cm, 1% methanol/chloroform) to obtain the foam title compound (22.0 g, 37 mmol, 72% yield). TLC: Rf= ($CHCl_3$/MeOH = 9/1).

[Example 25] Synthesis of Boc-L-Ab6-OTmse

Boc-L-Ab6-OH (620 mg, 2.0 mmol) and trimethylsilylethanol (0.572 ml, 4.0 mmol) were stirred in DCM (6 ml) for 6 hours in the presence of 4-dimethylamino-pyridine (24.4 mg, 0.2 mmol). The reaction solution was concentrated, dissolved in ethyl acetate, and successively washed with aqueous 10% citric acid solution, aqueous 4% sodium bicarbonate solution, and saturated

saline. The product was dried over $MgSO_4$ and concentrated, and the resulting oily substance was purified by flash silica gel chromatography (4x 30 cm, 10% ethyl acetate/hexane) to obtain an oily title compound (820 mg, 1.62 mmol, 81% yield). TLC: Rf= 0.97 ($CHCl_3$/MeOH = 9/1).

[Example 26] Synthesis of Boc-D-Tyr(Me)-L-Ile-D-Pro-L-Ab6-OTmse

Boc-D-Tyr(Me)-L-Ile-D-Pro-OBzl (1.01 g, 1.70 mmol) was dissolved in methanol (20 ml) and subjected to catalytic hydrogenation in the presence of 5% Pd-C (150 mg). After 8 hours, the catalyst Pd-C was filtered and the reaction solution was evaporated to obtain Boc-D-Tyr(Me)-L-Ile-D-Pro-OH.

Boc-L-Ab6-OTmse (1.51 g, 3.0 mmol) was dissolved in TFA (5 ml) and left standing on ice for 30 minutes. On completion of the reaction, the reaction solution was evaporated and the residue was vacuum-dried to obtain H-L-Am6-OTmse·TFA. This product was dissolved in DMF (3.5 ml). Under ice-cooling, Boc-D-Tyr(Me)-L-Ile-D-Pro-OH (819 mg, 1.62 mmol), HATU (776 mg, 2.0 mmol), and triethylamine (0.24 ml, 1.7 mmol) were divided into four aliquots and added to the above-described DMF solution, which was then stirred for 3 hours. The reaction solution was concentrated, dissolved in ethyl acetate, and successively washed with aqueous 10% citric acid solution, aqueous 4% sodium bicarbonate solution, and saturated saline. The resulting product was dried over anhydrous $MgSO_4$ and concentrated to obtain a foam substance, which was then purified by flash silica gel chromatography (4x 30 cm, 1% methanol/chloroform) to obtain the title compound (888 mg, 1.09 mmol, 64% yield). TLC: Rf= ($CHCl_3$/MeOH = 9/1).

[Example 27] Synthesis of Boc-L-Ab7-D-Tyr(Me)-L-Ile-D-Pro-OBzl

Boc-D-Tyr(Me)-L-Ile-D-Pro-OBzl (1.19 g, 2.0 mmol) was dissolved in TFA (5 ml) and left with standing on ice for 30 minutes. On completion of the reaction, TFA was removed by evaporation, and the residue was vacuum-dried to obtain H-D-Tyr(Me)-L-Ile-D-Pro-OBzl·TFA. The compound was dissolved in DMF

(4.0 ml), and Boc-L-Ab7-OH (652 mg, 2.0 mmol) was then added. HBTU (1.14 g, 3.0 mmol), HOBt·H_2O (306 mg, 2.0 mmol), and triethylamine (1.4 ml, 10 mmol) were further added and stirred for 3 hours under ice-cooling. The reaction solution was concentrated, dissolved in ethyl acetate, and successively washed with an aqueous 10% citric acid solution, an aqueous 4% sodium bicarbonate solution, and saturated saline. The product was dried over $MgSO_4$ and concentrated, and the resulting foam substance was purified by flash silica gel chromatography (4x 30 cm, 2% methanol/chloroform) to obtain the foam title compound (1.51 g, 1.89 mmol, 94% yield). HPLC retention time: 9.15 min.

[Example 28] Synthesis of Boc-L-Ab8-D-Tyr(Me)-L-Ile-D-Pro-OBzl

Boc-D-Tyr(Me)-L-Ile-D-Pro-OBzl (1.19 g, 2.0 mmol) was dissolved in TFA (5 ml) and left with standing on ice for 30 minutes. On completion of the reaction, TFA was removed by evaporation, and the residue was vacuum-dried to obtain H-D-Tyr(Me)-L-Ile-D-Pro-OBzl·TFA. The compound was dissolved in DMF (4.0 ml), and Boc-L-Ab8-OH (676 mg, 2.0 mmol) was then added. HBTU (1.14 g, 3.0 mmol), HOBt·H_2O (306 mg, 2.0 mmol), and triethylamine (1.4 ml, 10 mmol) were further added and stirred for 3 hours under ice-cooling. The reaction solution was concentrated, dissolved in ethyl acetate, and successively washed with an aqueous 10% citric acid solution, an aqueous 4% sodium bicarbonate solution, and saturated saline. The product was dried over $MgSO_4$ and concentrated, and the resulting foam substance was purified by flash silica gel chromatography (4x 30 cm, 2% methanol/chloroform) to obtain the foam title compound (1.44 g, 1.76 mmol, 88% yield). HPLC retention time: 10.9 min.

[Example 29] Synthesis of Boc-L-Ab9-D-Tyr(Me)-L-Ile-D-Pro-OBzl

Boc-D-Tyr(Me)-L-Ile-D-Pro-OBzl (1.19 g, 2.0 mmol) was dissolved in TFA (5 ml) and left with standing on ice for 30 minutes. On completion of the reaction, TFA was removed by evaporation, and the residue was vacuum-dried to obtain H-D-Tyr(Me)-L-Ile-D-Pro-OBzl·TFA. The compound was dissolved in DMF

(4.0 ml), and Boc-L-Ab9-OH (775 mg, 2.2 mmol) was then added. HBTU (1.14 g, 3.0 mmol), HOBt·H_2O (306 mg, 2.0 mmol), and triethylamine (1.4 ml, 10 mmol) were further added and stirred for 3 hours under ice-cooling. The reaction solution was concentrated, dissolved in ethyl acetate, and successively washed with an aqueous 10% citric acid solution, an aqueous 4% sodium bicarbonate solution, and saturated saline. The product was dried over $MgSO_4$ and concentrated, and the resulting foam substance was purified by flash silica gel chromatography (4x 30 cm, 2% methanol/chloroform) to obtain the foam title compound (1.31 g, 1.58 mmol, 79% yield). HPLC retention time: 11.7 min.

[Example 30] Synthesis of H-D-Tyr(Me)-L-Ile-D-Pro-L-Ab6-OH·TFA

Boc-D-Tyr(Me)-L-Ile-D-Pro-L-Ab6-OTmse (888 mg, 1.11 mmol) was dissolved in ethanol (10 ml). Under ice-cooling, an aqueous 1 N NaOH solution (1.32 ml, 1.33 mmol) divided into three aliquots was added to the solution and left standing on ice for 3 hours. The reaction solution was concentrated, dissolved in ethyl acetate, and successively washed with 10% citric acid and saturated saline. After drying over $MgSO_4$, the product was concentrated to obtain Boc-D-Tyr(Me)-L-Ile-D-Pro-L-Ab6-OH. The compound was dissolved in TFA (5 ml) and left standing on ice for 30 minutes. The reaction solution was evaporated, and the residue was vacuum-dried to obtain an oily title compound (778 mg, 1.07 mmol, 96% yield).

[Example 31] Synthesis of H-L-Ab7-D-Tyr(Me)-L-Ile-D-Pro-OH·TFA

Boc-L-Ab7-D-Tyr(Me)-L-Ile-D-Pro-OBzl (1.51 g, 1.89 mmol) was dissolved in methanol (5 ml) and subjected to catalytic hydrogenation in the presence of 5% Pd-C (150 mg). After 5 hours, the catalyst Pd-C was filtered and the reaction solution was evaporated to obtain Boc-L-Ab7-D-Tyr(Me)-L-Ile-D-Pro-OH. The resulting compound was dissolved in TFA (5 ml) and left standing on ice for 30 minutes. After the reaction solution was evaporated, the residue was vacuum-dried to obtain the oily title compound (1.15 mg, 1.84 mmol, 97% yield).

[Example 32] Synthesis of H-L-Ab8-D-Tyr(Me)-L-Ile-D-Pro-OH·TFA

Boc-L-Ab8-D-Tyr(Me)-L-Ile-D-Pro-OBzl (1.44 g, 1.76 mmol) was dissolved in methanol (5 ml) and subjected to catalytic reduction in the presence of 5% Pd-C (150 mg). After 5 hours, the catalyst Pd-C was filtered and the reaction solution was evaporated to obtain Boc-L-Ab8-D-Tyr(Me)-L-Ile-D-Pro-OH. The resulting compound was dissolved in TFA (5 ml) and left standing on ice for 30 minutes. After the reaction solution was evaporated, the residue was vacuum-dried to obtain the oily title compound (1.15 mg, 1.84 mmol, 97% yield).

[Example 33] Synthesis of H-L-Ab9-D-Tyr(Me)-L-Ile-D-Pro-OH·TFA

Boc-L-Ab9-D-Tyr(Me)-L-Ile-D-Pro-OBzl (1.31 g, 1.58 mmol) was dissolved in methanol (2 ml) and subjected to catalytic hydrogenation in the presence of 5% Pd-C (150 mg). After 12 hours, the catalyst Pd-C was filtered and the reaction solution was evaporated to obtain Boc-L-Ab9-D-Tyr(Me)-L-Ile-D-Pro-OH. This compound was dissolved in TFA (5 ml) and left standing on ice for 30 minutes. After the reaction solution was evaporated, ether/petroleum ether (1:10) was added to the residue for solidification. This was then vacuum-dried to obtain the title compound (905 mg, 1.42 mmol, 90% yield).

[Example 34] Synthesis of cyclo(-L-Ab6-D-Tyr(Me)-L-Ile-D-Pro-)

H-D-Tyr(Me)-L-Ile-D-Pro-L-Ab6-OH·TFA (778 mg, 1.07 mmol), HATU (616 mg, 1.62 mmol), and DIEA (0.75 ml) were divided into five aliquots and added to DMF (110 ml) every 30 minutes to carry out a cyclization reaction. After 2 hours, the solvent was removed by evaporation. The residue was dissolved in ethyl acetate, successively washed with 10% citric acid, 4% $NaHCO_3$, and saline. The product was dried over $MgSO_4$ and concentrated, and the resulting foam substance was purified by flash silica gel chromatography (4x 30 cm, 1% methanol/chloroform) to obtain a colorless oily compound (146 mg) (23%). HPLC retention time: 9.06 min; HRMS (FAB, dithiodiethanol), 579.2197 [M+H],

$C_{27}H_{41}O_5N_4{}^{79}Br$ (579.2182).

This time, a cyclic tetrapeptide containing an HOAt adduct transferred by substituting the Ab6 side chain terminus Br, cyclo(-L-A(OAt)6-D-Tyr(Me)-L-Ile-D-Pro-) (167 mg) (27%), was obtained as a foam. HPLC retention time: 8.16 min; HRMS (FAB, dithiodiethanol), 635.3312 [M+H], $C_{32}H_{43}O_6N_8$ (635.3306).

[Example 35] Synthesis of cyclo(-L-Ab7-D-Tyr(Me)-L-Ile-D-Pro-)

H-L-Ab7-D-Tyr(Me)-L-Ile-D-Pro-OH·TFA (1.15 g, 1.84 mmol), HATU (1.05 g, 2.76 mmol), and DIEA (1.28 ml) were divided into five aliquots and added to DMF (180 ml) every 30 minutes to carry out a cyclization reaction. The product was purified in the same manner as described above, resulting in a foam (700 mg) (64%). HPLC retention time: 9.90 min; HRMS (FAB, dithiodiethanol), 593.2300 [M+H], $C_{28}H_{42}O_5N_4{}^{79}Br$ (593.2339).

[Example 36] Synthesis of cyclo(-L-Ab8-D-Tyr(Me)-L-Ile-D-Pro-)

H-L-Ab8-D-Tyr(Me)-L-Ile-D-Pro-OH·TFA (512 mg, 0.80 mmol), HATU (455 mg, 1.20 mmol), and DIEA (0.56 ml) were divided into five aliquots and added to DMF (80 ml) every 30 minutes to carry out a cyclization reaction. After 2 hours, the reaction solution was concentrated, dissolved in ethyl acetate, and successively washed with aqueous 10% citric acid solution, aqueous 4% sodium bicarbonate solution, and saturated saline. This was then dried over $MgSO_4$ and concentrated, and the resulting foam substance was purified by flash silica gel chromatography (4x 30 cm, 1% methanol/chloroform) to obtain a foam (267 mg) (55%). HPLC retention time: 9.95 min; HRMS (FAB, dithiodiethanol), 607.2501 [M+H], $C_{29}H_{44}O_5N_4{}^{79}Br$ (607.2495).

[Example 37] Synthesis of cyclo(-L-Ab9-D-Tyr(Me)-L-Ile-D-Pro-)

H-L-Ab9-D-Tyr(Me)-L-Ile-D-Pro-OH·TFA (905 mg, 1.41 mmol), HATU (833 mg, 2.12 mmol), and DIEA (0.64 ml) were divided into five aliquots and added to DMF (150 ml) every 30 minutes to carry out a cyclic reaction. After two hours, the reaction solution was concentrated, dissolved in ethyl acetate, and

successively washed with aqueous 10% citric acid solution, aqueous 4% sodium bicarbonate solution, and saturated saline. This was then dried with $MgSO_4$ and concentrated, and the resulting foam substance was purified by flash silica gel chromatography (4x 30 cm, 1% methanol/chloroform) to obtain a foam (533 mg) (61%). HPLC retention time: 10.9 min; HRMS (FAB, dithiodiethanol), 621.2625 [M+H], $C_{30}H_{46}O_5N_4{}^{79}Br$ (621.2652).

[Example 38] Synthesis of cyclo(-L-Am6(Ac)-D-Tyr(Me)-L-Ile-D-Pro-)

Potassium thioacetate (57.6 mg, 0.504 mmol) was added to DMF (0.5 ml) containing cyclo(-L-Ab6-D-Tyr(Me)-L-Ile-D-Pro-) (146 mg, 0.252 mmol), and this was reacted for 3 hours. The reaction solution was concentrated, dissolved in ethyl acetate, and successively washed with aqueous 10% citric acid solution and saturated saline. After drying over $MgSO_4$ and concentrating, the resulting thioester was isolated and purified in the same manner as after the cyclic reaction to obtain an oily substance (114 mg) (79%). HPLC retention time: 9.06 min; HRMS (FAB, dithiodiethanol), 575.2879 [M+H], $C_{29}H_{43}O_6N_4S$ (575.2903).

[Example 39] Synthesis of cyclo(-L-Am7(Ac)-D-Tyr(Me)-L-Ile-D-Pro-)

Potassium thioacetate (52 mg, 0.452 mmol) was added to DMF (0.5 ml) containing cyclo(-L-Ab7-D-Tyr(Me)-L-Ile-D-Pro-) (133 mg, 0.226 mmol), and this was reacted for 3 hours. The reaction solution was concentrated, dissolved in ethyl acetate, and successively washed with aqueous 10% citric acid solution and saturated saline. After drying over $MgSO_4$ and concentrating, the resulting thioester was isolated and purified to obtain an oily substance (118 mg) (89%). HPLC retention time: 9.90 min; HRMS (FAB, dithiodiethanol), 589.3605 [M+H], $C_{30}H_{45}O_6N_4S$ (589.3060).

[Example 40] Synthesis of cyclo(-L-Am8(Ac)-D-Tyr(Me)-L-Ile-D-Pro-)

Potassium thioacetate (100 mg, 0.878 mmol) was added to DMF

(1 ml) containing cyclo(-L-Ab8-D-Tyr(Me)-L-Ile-D-Pro-) (267 mg, 0.439 mmol), and this was reacted for 3 hours. The reaction solution was concentrated, dissolved in ethyl acetate, and successively washed with aqueous 10% citric acid solution and saturated saline. After drying over $MgSO_4$ and concentrating, the resulting thioester was isolated and purified to obtain an oily substance (222 mg) (84%). HPLC retention time: 9.95 min; HRMS (FAB, dithiodiethanol), 603.3244 [M+H], $C_{31}H_{47}O_6N_4S$ (575.2903).

[Example 41] Synthesis of cyclo(-L-Am9(Ac)-D-Tyr(Me)-L-Ile-D-Pro-)

Potassium thioacetate (91.4 mg, 0.804 mmol) was added to DMF (0.5 ml) containing cyclo(-L-Ab9-D-Tyr(Me)-L-Ile-D-Pro-) (250 mg, 0.402 mmol), and this was reacted for 3 hours. The reaction solution was concentrated, dissolved in ethyl acetate, and successively washed with aqueous 10% citric acid solution and saturated saline. After drying over $MgSO_4$ and concentrating, the resulting thioester was isolated and purified to obtain an oily substance (190 mg) (77%). HPLC retention time: 10.9 min; HRMS (FAB, dithiodiethanol), 617.3364 [M+H], $C_{32}H_{49}O_6N_4S$ (617.3373).

[Example 42] Synthesis of cyclo(-L-Am6(-)-D-Tyr(Me)-L-Ile-D-Pro-) SS-dimer (SCOP 302)

Methanol (0.5 ml) containing cyclo(-L-Am6(Ac)-D-Tyr(Me)-L-Ile-D-Pro-) (114 mg, 0.198 mmol) was reacted with methanolic ammonia (10 eq.) to remove the acetyl groups. After the solvent was removed by evaporation, the residue was dissolved in DMF (2 ml), to which 1 M I_2 (ethanol) (0.2 ml) was added for oxidization. The SS-dimer produced was purified through a Sephadex LH-20 (DMF) column, and then mixed with water to obtain white powder. The yield was 82 mg (78%). HPLC retention time: 11.6 min; HRMS (FAB, dithiodiethanol), 1063.5391 [M+H], $C_{54}H_{79}O_{10}N_8S_2$ (1063.5361).

[Example 43] Synthesis of cyclo(-L-Am7(-)-D-Tyr(Me)-L-Ile-D-Pro-) SS-dimer (SCOP 304)

Methanol (0.5 ml) containing cyclo(-L-Am7(Ac)-D-Tyr(Me)-L-Ile-D-Pro-) (118 mg, 0.201 mmol) was reacted with methanolic ammonia to remove the acetyl groups of the compounds. The terminal sulfohydryl groups of the compounds were oxidized by adding 1 M I_2 (ethanol). After purification, SS-dimer was obtained as white powder. 98 mg (89%) yield. HPLC retention time: 12.3 min; HRMS (FAB, dithiodiethanol), 1091.5684 [M+H], $C_{56}H_{83}O_{10}N_8S_2$ (1091.5674).

[Example 44] Synthesis of cyclo(-L-Am8(-)-D-Tyr(Me)-L-Ile-D-Pro-) SS-dimer (SCOP 306)

Methanol (0.5 ml) containing cyclo(-L-Am8(Ac)-D-Tyr(Me)-L-Ile-D-Pro-) (222 mg, 0.368 mmol) was reacted with methanolic ammonia to remove the acetyl groups of the compounds. The terminal sulfohydryl groups of the compounds were oxidized by adding 1 M I_2 (ethanol). After purification, SS-dimer was obtained as white powder. 167 mg (81%) yield. HPLC retention time: 13.0 min; HRMS (FAB, dithiodiethanol), 1119.5961 [M+H], $C_{58}H_{86}O_{10}N_8S_2$ (1119.5987).

[Example 45] Synthesis of cyclo(-L-Am9(-)-D-Tyr(Me)-L-Ile-D-Pro-) SS-dimer (SCOP 308)

Methanol (0.5 ml) containing cyclo(-L-Am9(Ac)-D-Tyr(Me)-L-Ile-D-Pro-) (95 mg, 0.154 mmol) was reacted with methanolic ammonia to remove the acetyl groups of the compounds. The terminal sulfohydryl groups of the compounds were oxidized by adding 1 M I_2 (ethanol). After purification, SS-dimer was obtained as white powder. 84 mg (98%) yield. HPLC retention time: 14.2 min; HRMS (FAB, dithiodiethanol), 1147.6307 [M+H], $C_{60}H_{91}O_{10}N_8S_2$ (1147.6300).

[Example 46] Synthesis of cyclo(-L-Am7(SMEt)-D-Tyr(Me)-L-Ile-D-Pro-) (SS hybrid: SCOP 404)

DMF (0.5 ml) containing cyclo(-L-Am7(Ac)-D-Tyr(Me)-L-Ile-D-Pro-) (270 mg, 0.45 mmol) was reacted with methanolic ammonia (10 eq.) to remove the acetyl groups of the compounds. After

ammonia was removed by evaporation, 2-mercaptoethanol (10 eq.) and then 1 M I_2 (ethanol) 0.2 ml were added to the residue, causing oxidation. The resulting SS-hybrid was purified by a Sephadex LH-20 (DMF) column and freeze-dried to obtain the title compound as a white powder. The yield was 30 mg (11%). HPLC retention time: 8.9 min; HRMS (FAB, dithiodiethanol), 622.2877 [M], $C_{30}H_{46}O_6N_4S_2$ (622.2859).

[Example 47] Synthesis of cyclo(-L-Am7(S2Py)-D-Tyr(Me)-L-Ile-D-Pro-) (SS hybrid: SCOP 401)

DMF (1 ml) containing cyclo(-L-Am7(Ac)-D-Tyr(Me)-L-Ile-D-Pro-) (40 mg, 0.07 mmol) was mixed with 2,2'-dithiopyridine (31 mg, 0.14 mmol) and methanolic ammonia (10 eq.) and stirred for 8 hours. After the reaction solution was concentrated, the powder product was purified by flash silica gel chromatography (4x 30 cm, 1% methanol/chloroform) to obtain the title compound. The yield was 15 mg (38%). HPLC retention time: 9.6 min; HRMS (FAB, dithiodiethanol), 656.2952 [M+H], $C_{33}H_{45}O_5N_5S_2$ (656.2940).

[Example 48] Synthesis of cyclo(-L-Am7(S4Py)-D-Tyr(Me)-L-Ile-D-Pro-) (SS hybrid: SCOP 402)

DMF (1 ml) containing cyclo(-L-Am7(Ac)-D-Tyr(Me)-L-Ile-D-Pro-) (100 mg, 0.17 mmol) was mixed with 4,4'-dithiopyridine (75 mg, 0.34 mmol) and methanolic ammonia (20 eq.) and stirred for 8 hours. After the reaction solution was concentrated, the powder product was purified by flash silica gel chromatography (4x 30 cm, 1% methanol/chloroform) to obtain the title compound. The yield was 13 mg (13%). HPLC retention time: 6.5 min; HRMS (FAB, dithiodiethanol), 656.2934 [M+H], $C_{33}H_{45}O_5N_5S_2$ (656.2940).

[Example 49] Synthesis of cyclo(-L-Am7(SEll)-D-Tyr(Me)-L-Ile-D-Pro-) (SS hybrid: SCOP 403)

DMF (2.8 mL) containing 5,5'-dithiobis(2-nitrobenzoic acid) (515 mg, 1.4 mmol) was mixed with dimethylamine (343 mg, 3.0 mmol), DCC (867 mg, 3.0 mmol), and HOBt·H_2O (214 mg, 1.4 mmol), and then stirred for eight hours while cooling on ice. On

completion of the reaction, the reaction solution was concentrated, dissolved in ethyl acetate, and successively washed with an aqueous 10% citric acid solution, an aqueous 4% sodium bicarbonate solution, and saturated saline. After drying over $MgSO_4$, ethyl acetate was removed by evaporation. The residue was vacuum-dried and purified using flash silica gel chromatography (4x 30 cm, 1% methanol/chloroform) to obtain 5,5'-dithiobis(2-nitrobenzoic acid dimethylamide).

DMF (2 ml) containing cyclo(-L-Am7(Ac)-D-Tyr(Me)-L-Ile-D-Pro-) (130 mg, 0.22 mmol) was mixed with 5,5'-dithiobis(2-nitrobenzoic acid dimethylamide) (198 mg, 0.44 mmol) and methanolic ammonia (10 eq.), and then stirred for 6 hours. The reaction solution was concentrated, dissolved in a small amount of DMF, and purified by HPLC (column: YMC-Pack ODS-A 10x 250 mm) to obtain the title compound. The yield was 13 mg (9.3%). HPLC retention time: 9.5 min; HRMS (FAB, dithiodiethanol), 771.3201 [M+H], $C_{37}H_{50}O_8N_6S_2$ (771.3210).

[Example 50] Synthesis of cyclo(-L-Am7(SMe)-D-Tyr(Me)-L-Ile-D-Pro-) (SCOP 405)

DMF (1 ml) containing cyclo(-L-Ab7-D-Tyr(Me)-L-Ile-D-Pro-) (118 mg, 0.2 mmol) was mixed with 4-methoxybenzylmercaptan (0.056 ml, 0.4 mmol) and triethylamine (0.07 ml, 0.5 mmol) and reacted at a room temperature for 2 hours. The produced cyclo(-L-Am7(Mb)-D-Tyr(Me)-L-Ile-D-Pro-) was extracted with ethyl acetate, purified, and then reacted with dimethyl(methylthio)sulfonium tetrafluoroborate (0.9 mmol, 176 mg) in methanol (18 ml) at room temperature for 2 hours. The reaction solution was concentrated, dissolved in chloroform, and purified by silica gel chromatography (2x 25 cm, 2% methanol/chloroform) to obtain the target product. The yield was 69 mg (65%). TLC Rf: 0.90 ($CHCl_3$/MeOH = 19/1). HPLC retention time: 12.28 min; HR-FAB+ MS: 593.2777 (calcd.: 592.2753, composition: $C_{29}H_{44}O_5N_4S_2$, matrix: 2,2'-ditihidiethanol).

[Example 51] Measurement of HDAC inhibition activity

In this Example, the HDAC inhibition activity of SCOP was measured. Fig. 1 to Fig. 4 show lists of the structures of the sulfur-containing cyclic peptides (SCOP) whose activity was measured. The conformation and number of carbon chains until the active groups of the cyclic tetrapeptide structures were investigated based on natural HDAC inhibitors, Cyl-1 and Cyl-2, as shown in Fig. 5 (Furumai et al. (2001) Proc. Natl. Acad. Sci. USA, 98, 87-92).

The steric conformation of natural Cyl-1 and Cyl-2 is LDLL, however those with LDLD conformation were also investigated. In the following experimental results, DTT is coexisted for X = H, for the purpose of cutting disulfide bonds.

To measure HDAC inhibition activity, an HDAC solution was prepared as described below. 1x 10^7 of 293T cells were plated on to a 100-mm dish and, after 24 hours, transfected with vectors (1 μg) expressing human HDAC1 and HDAC4 or mouse HDAC6, using LipofectAmine 2000 reagent (Life Technologies, Inc. Gaithersburg, MD). The above-mentioned pcDNA3-HD1 was used as a vector expressing human HDAC1 (Yang, W. M., Yao, Y. L., Sun, J. M., Davie, J. R. & Seto, E. (1997) J. Biol. Chem. 272, 28001-28007). pcDNA3.1(+)-HD4 was used as a vector expressing human HDAC4 (Fischle, W., Emiliani, S., Hendzel, M. J., Nagase, T., Nomura, N., Voelter, W. & Verdin, E. (1999) J. Biol. Chem. 274, 11713-11720). pcDNA-mHDA2/HDAC6 was used as a vector expressing mouse HDAC6 (Verdel, A. & Khochbin, S. (1999) J. Biol. Chem. 274, 2440-2445). The vectors were introduced for five hours in OPTI-MEM. The medium was then replaced with Dulbecco's modified Eagle's medium (DMEM), and incubated for 19 hours. The cells were washed with PBS, suspended in lysis buffer (50 mM Tris-HCl (pH7.5), 120 mM NaCl, 5 mM EDTA, and 0.5% Nonidet P-40), and sonicated. The supernatant was collected by centrifugation and nonspecific protein was removed using Protein A/G plus agarose beads (Santa Cruz Biotechnologies, Inc.). Anti-FLAG M2 antibodies (Sigma-Aldrich, Inc.) were added to the supernatant of cells expressing HDAC1 or HDAC4. Anti-HA antibodies (clone 3F10, Roche Molecular Biochemicals) were added to the

supernatant of cells expressing HDAC6. Reaction in the respective mixtures was carried out at 4°C for one hour. The resulting reaction mixtures were independently mixed with agarose beads and further reacted at 4°C for one hour. The agarose beads were washed three times with lysis buffer and then washed once with HD buffer (20 mM Tris-HCl (pH8.0), 150 mM NaCl, 10% glycerol, and a complete protease inhibitor cocktail (Boehringer Mannheim, Germany)). The protein solution, referred to as "HDAC reaction solution", that had bonded to the agarose beads was recovered by incubation with FLAG peptide (40 μg) (Sigma-Aldrich, Inc.) or HA peptide (100 μg) in an HD buffer (200 μl) at 4°C for one hour. The HDAC reaction solution was used for determining HDAC inhibition activity as shown below.

In vitro HDAC inhibition activity was evaluated as follows: A test compound was dissolved in DMSO and adjusted to 10 mM. This was used as an inhibitor stock solution. As a positive control, Tricostatin A (TSA), known as an HDAC inhibitor, was dissolved in DMSO to obtain a 10 mM stock solution. Measurement was carried out by incubating each of the above-mentioned HDAC solutions and a solution of acetylated histone substrate labeled with [^{3}H] at 37°C for 15 minutes (100 μl reaction volume) in the presence of a test compound or control TSA. These reactions were stopped by adding 10 μl HCl. The [^{3}H] acetic acid excised by the enzyme reaction was extracted with ethyl acetate and subjected to radioactive dose measurement. As a negative control, the same procedure was carried out in which no inhibitor was added to the reaction system. The inhibition activity was expressed as a 50% inhibition concentration ("IC50 (nM)") of the HDAC activity in the negative control (Tables 1 to 4).

The HDAC inhibition activity *in vivo* was measured using p21 promoter-inducing activity as an index, as shown below. The MFLL-9 cells employed for the experiments stably maintained fusion genes of human wild-type p21 promoter and luciferase (Dr. B. Vogelstaein). Using phenol red-free DMEM medium comprising 10% FBS, cultivation was carried out in a steam-saturated incubator at 37°C with 5% carbon dioxide. The MFLL-9 cells were

plated at a density of 85,000 cells/well on a 96-well microtiter plate, each in 99 µl of the above-mentioned medium. These were then cultivated for six hours. One µl of test compound solution was added to each well, which was then cultured for another 18 hours. TSA was used as the positive control compound with p21 promoter-inducing activity, which results from HDAC inhibition activity.

The intensity of luminescence caused by the product of the enzyme reaction for intracellular luciferase expression was measured using Luc Lite (Packard BioScience Company). A group in which test compounds were not added was used as a negative control group. The values measured for this group were used as a standard. The activities for each concentration of added test compound were expressed relative to the above-mentioned standard value, 1. The test compound activity intensities were compared using the concentrations ("EC50 (nM)") corresponding to 50% of the maximum active values for TSA (Tables 1 to 4).

Table 1

X = H (coexisting with DTT)

Inhibitor	IC50 (nM)			P21 Promoter		
SCOP No.	HDAC1	HDAC4	HDAC6	EC50 (nM)	Conformation	Number of Carbon Chains
148	81.4	17.0	>500000	6720	Cyl1 (LDLL)	C5
149	2.37	5.22	44300	596	Cyl2 (LDLL)	C5
150	2.10	4.26	5560	504	Cyl2 (LDLD)	C5
151	932	7340	28500	>100000	Cyl1 (LDLD)	C4
152	4.60	2.06	1400	309	Cyl1 (LDLD)	C5
153	9.13	91.0	8050	9850	Cyl1 (LDLD)	C6
154	38.1	99.2	2470	31400	Cyl1 (LDLD)	C7

As the *in vitro* inhibition activity and *in vivo* P21 promoter activity of Table 1 shows, LDLD isomers were found to have higher activities than LDLL isomers which are in a natural conformation. In addition, C5 was shown to be most preferable as the number of carbon chains until the active thiol group.

Table 2

X = a compound comprising the left conformation (homodimer)

Inhibitor	IC50 (nM)			P21 Promoter		
SCOP No.	HDAC1	HDAC4	HDAC6	EC50 (nM)	Conformation	Number of Carbon Chains
296	763	222	>500000	7730	Cyl1 (LDLL)	C5
298	114	33.7	418000	5800	Cyl2 (LDLL)	C5
300	61.1	36.2	255000	7370	Cyl2 (LDLD)	C5
302	7200	>500000	>500000	>100000	Cyl1 (LDLD)	C4
304	142	145	>500000	341	Cyl1 (LDLD)	C5
306	153	319	1320000	847100000	Cyl1 (LDLD)	C6
308	983	505	745000	235000	Cyl1 (LDLD)	C7

As for the case of X = H, with respect to the homodimers, it was shown that LDLD isomers have higher activities than LDLL isomers which are in a natural conformation, and that C5 is the most preferable number of carbon chains until the active thiol group.

Table 3

X = a low molecular-weight compound (hybrid)

Inhibitor	IC50 (nM)			P21 Promoter	
SCOP No.	HDAC1	HDAC4	HDAC6	EC50 (nM)	Conformation
401	NT	NT	NT	1360	152 + 2-Pyridine
402	6.76	68.3	1610	1310	152 + 4-Pyridine
403	21.5	18.9	6080	1800	152 + Ellman's reagent
404	217	355	201000	1360	152 + Mercaptoethanol
405	119	405	191	3260	152 + Methylmercaptane
401/DTT	NT	NT	NT	815	
402/DTT	0.553	1.12	2010	470	
403/DTT	1.15	1.53	4730	748	
404/DTT	2.44	13.0	15400	754	

"NT" means that no test was carried out.

Even hybrid bodies of SCOP 152 and low molecular-weight compounds were shown to have inhibition activity.

Table 4

Positive control (TSA)

Inhibitor	IC50 (nM)			P21 Promoter
TSA	HDAC1	HDAC4	HDAC6	EC50 (nM)
TSA	19.2	68.3	27.2	445

According to the above results, LDLD isomers have higher activities than LDLL isomers in their natural conformation. In addition, C5 was found to be the most preferable number of carbon chains until the active thiol group. Furthermore, since the HDAC6 inhibition activity was significantly low, the compounds were confirmed to comprise enzyme subtype-selective

inhibition activity. On an enzymatic level, the present compounds showed high HDAC inhibition activity when they were thiols (X = H) coexisting with DTT. However, on a cellular level, even those X = left structure or X = low molecular-weight compounds showed high activity. This suggests that thiol groups were exposed and thus activated by reducing the disulfides incorporated into cells using intracellular reducing forces.

Next, since it was possible that DTT had some effect, an experiment was carried out using purified SCOP 152, under DTT-free conditions.

Table 5

	IC50 (nM)			P21 Promoter
Inhibitor	HDAC1	HDAC4	HDAC6	EC50 (nM)
152	NT	NT	NT	3510

"NT" means that no test was carried out.

The EC50 value increased compared to when DTT was present. DTT's existence was thought to reduce the pH of the culture medium, causing a change in monomer stability. Alternatively, it may be also possible that DTT served as a protection group. The following experiment was carried out using purified SCOP 152 under the DTT-free conditions.

[Example 52] Measurement of *in vivo* HDAC inhibition activity

Histone acetylation levels were measured by: (i) reacting a test compound with HeLa cells; and (ii) confirming the histone acetylation level by Western blotting using anti-acetylated lysine antibodies. Specifically, human uterine cancer cells (HeLa) were cultured in a DMEM medium comprising 10% FBS at 37°C in the presence of 5% carbon dioxide in a steam-saturated incubator. Two ml of the cells at a density of 15,000 cells/ml were plated onto a 6-well plate and cultured for 18 hours. Test compound solution was added to each culture and successively cultured for another six hours. The cells were washed with PBS,

suspended in a lysis buffer (50 mM Tris-HCl (pH7.5), 120 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40), and then sonicated. The supernatant was collected by centrifugation, mixed with SDS buffer, and left at 100°C for five minutes. The resulting sample was subjected to electrophoresis on a 15% SDS gel and transferred to a membrane film. This was treated with primary antibody "AKL5C1" (Japan Energy), and secondary antibody "anti-mouse" (LIFE SCIENCE), and then acetylation bands were detected by ECL (amersham pharmacia biotech) (Fig. 6). The concentration unit of the compounds shown in Fig. 6 is "nM".

As shown in Fig. 6, the inhibition tendencies shown were the same as the results (EC50) of P21 promoter-inducing activity. C5 was the most preferable number of carbon chains until the active thiol group.

[Example 53] Cytotoxicity test

Human normal lung cells (TIG-3) and human uterine cancer cells (HeLa) were used to test SCOP cytotoxicity. These TIG-3 and HeLa cells were cultured in a DMEM medium comprising 10% FBS at 37°C in the presence of 5% carbon dioxide in a steam-saturated incubator. The TIG-3 and HeLa cells were plated in 100 µl/well of the above-described medium on a 96-well microtiter plate, at a density of 30,000 cells/well and 10,000 cells/well respectively. This was then cultured for 18 hours. Test compound solution diluted with medium was added to each well and culture was continued for another 48 hours.

30 µl of supernatant from each well was transferred to another 96-well microtiter plate (A), and the remaining supernatant was discarded. 100 µl of 0.5% Triton-X/PBS was added to each well to lyse the cells, and 30 µl was then transferred to each respective well of another 96-well microtiter plate (B). 30 µl of LDH-Cytotoxic Test (Wako) substrate solution was added to each well of these 96-well microtiter plates A and B, causing a color reaction. Once the color reaction was sufficiently progressed, it was stopped by adding 60 µl of a quenching solution. Color intensity was measured at OD560 nm using a

microplate reader (Softmax). [A/(A+B)] was calculated as the free-LDH ratio. Inhibition activity was shown as LD50 when the free-LDH ratio was 50%. The higher the activity value for cancer-cell-selective cell damage (LD50 for normal cells/LD50 for cancer cells), the more that cancer-cell-selective apoptosis was induced.

Table 6

Inhibitor	LD50(nM)		Cancer-Cell-Selective Cytotoxicity
	HeLa	TIG-3	
TSA	41.4	1580	38.2
SCOP 152	370	6780	18.3
SCOP 304	151	3471	23.0
SCOP 402	1170	13300	11.4
SCOP 405	179	7900	44.1
SCOP 304/DTT	47.1	1190	25.2
SCOP 402/DTT	161	4460	27.8

As shown in Table 6, the compounds of the present invention were confirmed to have intense cancer-cell-selective cytotoxicity that is as effective as TSA.

[Example 54] Evaluation of Stability

The stability of SCOP 152, SCOP 304, and SCOP 402 in serum was evaluated by the method shown below: 1 µl of 10 mM SCOP 152, SCOP 304, and SCOP 402 was added to 99 µl FCS, and incubated at 37°C. Each hour, NaCl in a sufficient amount for saturation and 1 ml of ethyl acetate were added to each mixture. After extraction, from 800 µl of the ethyl acetate phase, the ethyl acetate was distilled off, and then 100 µl of DMSO was added to the residue. The resulting solution was further diluted ten times with DMSO and used to measure p21 promoter inducing activity. Activity at incubation time zero was taken as 100%,

and activities were compared (Fig. 7).

As shown in Fig. 7, SCOP 304 and SCOP 402 were able to stably retain activity in serum for longer than SCOP 152. This stability was thought to be improved by the protection of thiol groups.

Next, *in vivo* stability was investigated based on histone acetylation levels. HeLa cells were treated with each compound and then histone acetylation level was analyzed by Western blotting using an anti-acetylated lysine antibody (Fig. 8). Specifically, human uterine cancer cells (HeLa) were cultured in DMEM medium comprising 10% FBS at 37°C with 5% carbon dioxide in a steam-saturated incubator. Two ml of the cells were plated at a density of 15,000 cells/ml in a 6-well plate, and cultured for 18 hours. 200 nM of test compound solutions comprising TSA, SCOP 152 and SCOP 304, and 1 µM test compound SCOP 402 solution were added, and culture was continued for an appropriate time. The cells were washed with PBS, suspended in a lysis buffer (50 mM Tris-HCl (pH7.5), 120 mM NaCl, 5 mM EDTA, and 0.5% Nonidet P-40), and then sonicated. Each supernatant was collected by centrifugation, mixed with a SDS buffer, and treated at 100°C for five minutes. The resulting sample was subjected to electrophoresis on a 15% SDS gel and transferred to a membrane film. After treatment with primary antibody "AKL5C1" (Japan Energy), and secondary antibody "anti-mouse" (LIFE SCIENCE), ECL (amersham pharmacia biotech) treatment was carried out and acetylation bands were detected.

The compounds of the present invention showed intense inhibition activity towards HDAC1 and HDAC4, but scarcely any inhibition activity towards HDAC6. HDAC6 is highly expressed in the testes and such, and is predicted to be relevant to normal tissue differentiation. However, HDAC6 has not been found to be related to carcinogenesis. Therefore, inhibition of HDAC6 may lead to side effects. Since the compounds of the present invention have extremely weak HDAC6 inhibition activity, as well as sub-type selectivity, which TSA does not have, they are useful as novel inhibitors. Furthermore, the tetrapeptide

backbone structure of the compounds of the present invention can be easily changed, suggesting further selectivity can be conferred.

Industrial Applicability

As described above, the compounds of the present invention show strong selective inhibitory activity towards HDAC1 and HDAC4. Accordingly, the compounds of the present invention may be useful as pharmaceutical agents for treating or preventing diseases associated with HDACs, particularly HDAC1 and HDAC4. The methods for producing the compounds of the present invention are carried out by using 2-amino-n-haloalkanoic acid as a raw material to easily synthesize various types of compounds. Consequently, use of the production methods of the present invention is expected to contribute to the development of HDAC inhibitors with greater selectivity.